The production and study of *Theileria annulata* macroschizont infected cells: relating to MHC class II expression, T cell stimulatory ability, cytokine mRNA production and their use as vaccines.

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Declaration.

I hereby declare that the work presented in this thesis is the product of my own efforts and has not been submitted in any previous application for another degree. The work on which this thesis is base is my own except where stated in the text or acknowledgements.

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Abstract.

Theileria annulata is a protozoan parasite of cattle of the genus Apicomplexa, which causes the life threatening disease Tropical Theileriosis. In susceptible animals progression of this disease can be rapid with death occurring within 14 to 20 days post infection. *T.annulata* infection is characterised by the proliferation of macrophages (Mθs) infected with the macroschizont stage of the parasite's life cycle. This stage is closely associated with pathology but may also be the main stage against which animals mount a protective response.

The aims of this thesis are to (1) examine the levels of MHC class II on infected cells, (2) attempt to relate MHC class II expression to their T cell stimulatory ability, (3) investigate the cytokine mRNA expression of infected cells and ascertain correlations between this and the T cell reactions they induced, (4) study the relationships between the cytokine mRNAs produced by infected cells and their potential effects on pathogenesis. *In vitro*, *T.annulata* infected cells possess augmented antigen presenting function and also the ability to activate resting naive/memory autologous T cells in a contact dependent manner. *In vivo* infected cells have been shown to congregate initially in the medulla of the draining lymphnode, where they associate with and activate T cells.

This thesis investigates T cell stimulatory ability of *T.annulata* infected cells. Clonal populations of *T.annulata* infected cells were generated from $CD14^+$ monocytes and Mθs, cultured and used to stimulate autologous naive T cells. T cell proliferation assays showed the clonal cultures possessed different T cell stimulatory abilities. Previous work showed that infected cells expressed elevated levels of MHC class II molecules. These molecules are involved in the stimulation of T cells and the possibility that the signals involved in nonspecific T cell activation were linked to MHC class II expression was investigated. Expression levels of MHC class II molecules by the clonal cultures did not correlate with the T cell stimulatory ability of the infected cells. After this finding the production of cytokine mRNA by infected cells was investigated. Cytokines are known to play major roles in the control of cellular activation/proliferation and immune responses.

During this study the production of cytokine mRNA specific for: IL-1α; IL-1β; IL-2; IL-4; IL-6; IL-10; TNFα and IFNγ by clonal populations of infected cells were investigated using reverse transcriptase polymerase chain reaction (RT-PCR). Experiments showed that parasitised cells produce cytokine mRNAs typically associated with cells of the monocyte/Mθ lineage. Limiting cycle RT-PCR was then employed to study differences in the levels of cytokine mRNAs produced by the different infected cell populations. This showed that variation in the T cell proliferation induced by the various populations of infected cells positively correlated with the levels of T cell stimulatory cytokine mRNAs produced by the infected cells. T cell proliferation assays showed the *in vitro* nonspecific T cell response peaks at day 5 post stimulation. The cytokine profiles of the responding T cells were then investigated from days 1 to 7. It was found that the T cell response following stimulation with infected cells always skewed to a Th_1 like response and that the differences in induced proliferation did not correlate with the levels of IL-2 and IL-4 produced by the responding populations. This suggested that infected cells possess the ability to manipulate T cell activity and that this would appear to occur via infected cell/T cell contact and T cell stimulatory cytokine production by the infected cells. Study also showed that infected cells initiate a none protective Th_1 like response, before leaving the node to enter the periphery.

The main method of control of *T.annulata* in many areas of the developing world is the immunisation of animals with attenuated *T.annulata* infected cell line vaccines. Long term *in vitro* culture of infected lines has been employed to produced successful vaccines. The mechanisms of attenuation are poorly understood. Following the findings that cytokine production by infected cells may have important consequences with respect to the induction of pathology and the control of immune responses during *T.annulata* infection, a clinical trial was performed to assess the efficiency of two of the clonal cultures as cell line vaccines. The two clones chosen (clones I and L) differed greatly in the levels of T cell stimulatory cytokine mRNA they produced and the levels of T cell proliferation they induced. Clone I induced low levels of T cell proliferation and produced low levels of cytokine mRNA, whilst clone L induced high levels of T cell proliferation and produced higher levels of cytokine mRNA. After inoculation of three animals with each of these cell lines and analysis of the data

obtained (temperature, red/white blood cell counts, packed cell volume) it was found that the severity of the infection induced by clone L was greater than that induced by clone I. However, challenge of these animals some 60 days later showed all six to be equally resistant to infection with a lethal dose of *T.annulata* sporozoites.

In summary, this thesis shows that the levels of T cell stimulatory cytokines produced by *T.annulata* infected cells correlate with the proliferative response of autologous T cells cultured with infected cells but that the levels of T cell proliferation do not correlate with the levels of autocrine cytokines produced by the proliferating T cells. The findings of this study also suggest that assessment of T cell stimulatory ability and cytokine mRNA production can aid in the selection of putative cell line vaccines.

Chapter I.

Theileria annulata, Tropical Theileriosis and The Immune System.

Introduction.

In the developing world many farmers continue to operate on a subsistence basis (*eg*, 80% of African farmers (Innes *et al*, 1992)). Cattle are of high importance to many of these farmers and especially european breeds very much so due to the low productivity of local breeds of cattle (Pino, 1981). The practice of importing exotic european breeds is attractive to many governments as a method of improving productivity (Gill *et al*, 1980).

A serious drawback to these attempts to improve stock has proved to be tick borne diseases such as theileriosis and babesiosis (Uilenberg *et al*, 1993). This thesis concerns tropical theileriosis, the disease caused by the protozoan parasite *Theileria annulata*. A study in 1982 estimated that in excess of 250 million cattle were at risk from tropical theileriosis world wide (Robinson, 1982). Investigations have also shown the disease to be a significant burden upon the economies of developing countries, *e.g.* losses of at least $15 million per annum in Morocco (R.L.Spooner, pers. comm).

T.annulata is transmitted by ticks of the genus *Hyalomma*, whose distribution ranges from southern Europe, the USSR and the mediterranean littoral to China and The Far East (Purnell, 1978; Robinson, 1982). Entry of the infective stage of the parasite (the sporozoite) into the host is via a tick bite, with the parasite then localising in the draining lymph node. Within the node the parasite develops into the macroschizont stage which resides within host leucocytes. Infected cells proliferate rapidly, with infected cells then moving to the peripheral circulation and infiltrating many host tissues (Neitz, 1957; Barnett, 1977). The parasite is able to pass back into feeding ticks after macroschizont infected cells produce microschizonts, which give rise to merozoites. After release merozoites invade erythrocytes forming the piroplasm stage of the life cycle, infecting ticks after ingestion and forming new sporozoites within the

tick salivary glands.

Infected susceptible cattle suffer a range of symptoms following infection. These include: fever; anaemia; respiratory distress and cachexia (Barnett, 1977). There is some mortality and morbidity in endemic cattle but by far the worst problems occur when susceptible exotic cattle are introduced into areas where *T.annulata* is present (Hashemi-Fesharki, 1988; Uilenberg *et al*, 1993). The mortality rates recorded in exotic cattle vary between 40-70% (Uilenberg *et al*, 1993; Brown, 1990) but can be as high as 90%, severely reducing the productivity of affected areas (Robinson, 1982; Hashemi-Fesharki, 1988).

It is possible to control infection by the use of accaracides (controlling tick numbers) and to protect susceptible animals by immunisation with attenuated infected cell line vaccines (Pipano, 1981; Hashemi-Fesharki, 1988) and chemotherapy (McHardy *et al*, 1985; Dhar *et al*, 1990) but these approaches are expensive, time consuming and prove an extra burden upon developing areas.

Vaccine production and dosage have not been standardised, with methods of production and immunisation protocols varying from country to country, with groups sometimes using large cell doses to produce protection (Adalar *et al*, 1994). There are a number of drawbacks to cell line immunisation which include: production and distribution of vaccines; the waning of immunity if animals are not challenged (Pipano, 1977; Baylis *et al*, 1993); revaccination with the same cell line results in a reduction in protection (Ouhelli *et al*, 1994; Nichani *et al*, 1997). These problems show that further work into vaccine development and production remains to be carried out.

There has been much research into the behaviour of the parasite *in vitro* and has led to the discovery of a number of interesting characteristics of infected cells. It was shown *in vivo* that only macroschizont infected cells need to be present to induce all the symptoms of tropical theileriosis (Hooshmand-Rad, 1976) and *in vitro* studies have demonstrated that these rapidly proliferating cells can have dramatic effects upon autologous T cells when cultured together (Glass *et al*, 1990; Campbell *et al*, 1996). It was found that infected cells generally expressed high levels of Major histocompatibility complex (MHC) class II molecules and that when T cells from

naive animals were cultured with macroschizont infected cells they were induced to proliferate (Glass *et al*, 1990; Rintelen *et al*, 1990; Spooner and Glass, 1990a). This T cell activation is contact specific and can be blocked by the presence of anti MHC class II or anti CD4 mAbs (Campbell, pers. comm.).

The infected cells also caused responding T cells to always produce a T helper type 1 cytokine response, producing high levels of Interleukin 2 (IL-2) and Interferon gamma (IFNγ) but low levels of Interleukin 4 (IL-4) (Nichani, 1994; Campbell, 1995). The mechanism by which responding T cells become activated is unknown but work by Campbell *et al* (1995) showed that T cell activation and proliferation was contact dependent and Preston *et al* (1992) found high levels of macrophage (Mθ) associated cytokines (such as Tumour necrosis factor α (TNFα) and Interleukin 1 (IL-1)) present when peripheral blood mononuclear cells (PBM) were incubated with infected cells. These cytokines appeared to have no deleterious effect upon infected cells and it was speculated that they may exacerbate theilerial infections and possibly enhance the growth of infected cells (Preston, Brown and Richardson, 1992). High levels of cytokines such as these are observed in cases of septic shock (Waage *et al*, 1989; Creasy *et al*, 1991). This may relate to a number of the symptoms observed during tropical theileriosis, such as, cachexia, fever and respiratory distress.

This study investigates the roles of MHC class II and cytokine expression by cells infected with *T.annulata* in the immunopathology of tropical theileriosis. The phenomenon of non-specific T cell activation by infected cells and the possibility that damage observed in the lymph nodes of animals undergoing infection may be due to inappropriate stimulatory abilities of infected cells is investigated.

The aims of this study are:

(1) To quantify the levels of MHC class II expression by infected cells.

(2) To attempt to relate the MHC class II expression to the T cell stimulatory ability of the infected cells.

(3) To investigate cytokine mRNA expression of infected cells and ascertain correlations between this, T cell stimulatory ability and the T cell reactions induced.

(4) To study the relationships between cytokine mRNA production by infected cells and the ability of cell lines to act as vaccines.

Section 1.0

Literature Review.

***Theileria annulata*, the parasite, infection and immunity.**

This thesis investigates the host response to the protozoan parasite *Theileria annulata.* The parasite infects cattle, causing the disease Tropical theileriosis and induces high levels of mortality and morbidity within endemic areas. This study relates to the infection of exotic animals (nonindigenous species of cattle) by this parasite and to *in vivo* reactions post infection.

Section 1.1.

The taxonomy of *Theileria spp.*

The protozoan parasite *T.annulata* was first discovered in 1904, in Russia by Dschunkowsky and Luhs (Melhorn & Schein, 1984). Despite the fact that both piroplasm and schizont forms of *T.parva* were probably observed by Koch, *T.annulata* and *T.parva* were originally named *Piroplasma* (reviewed by Norval *et al*, 1992). Work by Bettencourt *et al*, resulted in renaming them as *Theileria* in 1907.

Taxonomic studies have revealed 39 species of *Theileria* (Levine *et al*, 1988), however, the actual number is almost certainly lower, with a number of the species names being synonyms (Uilenberg, 1981). Because *T.annulata* and *T.parva* induce the most pathology, research into the *Theileridae* has largely concentrated on these two parasites.

Table 1.0.

Kingdom.	Protista.
Subkingdom.	Protozoa.
Phylum.	Apicomplexa.
Class.	Sporozoea.
Subclass.	Piroplasmia.
Order.	Piroplasmida.
Family.	Theileridae.
Genus.	Theileria.

Table 1.0 Taxanomic classification of *Theileria spp.*

The table above is adapted from Irvin (1987) and Norval *et al*, (1992) and shows the taxanomic classification of *Theileria Spp* from kingdom to genus.

T.annulata and *T.parva* share a common lineage with malarial parasites but diverge from these related protozoans to form the Piroplasmida. This divergence in classification is due to the lack of crystalloid and pigment in the Piroplasmida, as opposed to their presence in the Eucoccidian malarial parasites. There are only two types of organisms in this order, which are *Theileria* and *Babesia* (Barnett, 1977).

Section 1.2.

Geographical distribution of the *T.annulata* and *T.parva*.

Areas affected by *T.annulata* range from across the mediterranean littoral, Southern Europe, North Africa, southern former USSR, to the Middle East, India, China and The Far East (Purnell, 1978; Robinson, 1982), as shown in the Diagram 1.

T.parva has a much more restricted distribution, being present only in Africa. Within this continent affected areas are concentrated around the Eastern coast but the parasite has also been detected in central and southern regions (Norval, 1992; Uilenberg *et al*, 1993). The territories affected by these two parasites only overlap in the Sudan (Norval 1992).

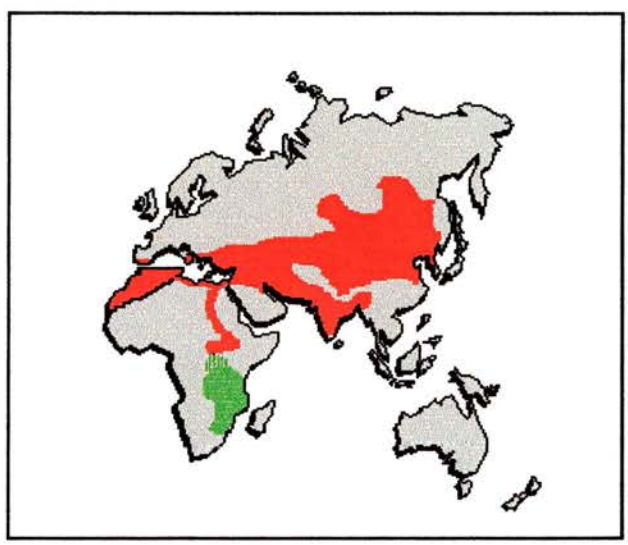


Diagram 1. The geographical distributions of *T.annulata* and *T.parva*.

A map showing the distibutions of *T.annulata* (shown in red) and *T.parva* (shown in green).

The reasons for the differences in the distributions are not fully known, however, they are probably related to the distributions of their respective invertebrate hosts. These two Theileria species are transmitted to their bovine hosts by ticks, with the two parasites favouring different tick species as vectors. *T.annulata* has been shown to be transmitted exclusively by ticks of the genus *Hyalomma* (Purnell, 1978), whilst *T.parva* is mainly transmitted by ticks of the genus *Rhipicephalus* (Norval, 1992) but can also be transmitted by some *Hyalomma* ticks. Robinson demonstrated in 1982 that 15 species of *Hyalomma* ticks were capable of transmitting *T.annulata* but the most widely recognised vector species are *H. anatolicum anatolicum* and *H. detritum*. These ticks both have wide distributions, with *H. a. anatolicum* being present from North West Africa through to India, with the distribution of *H. detritum* ranging from the Far East, India to the former USSR (Norval, 1992). The main cause of the differences in distribution of these protozoan parasites is therefore probably the differences in vector distribution.

Section 1.3.

Life cycle of *T.annulata.*

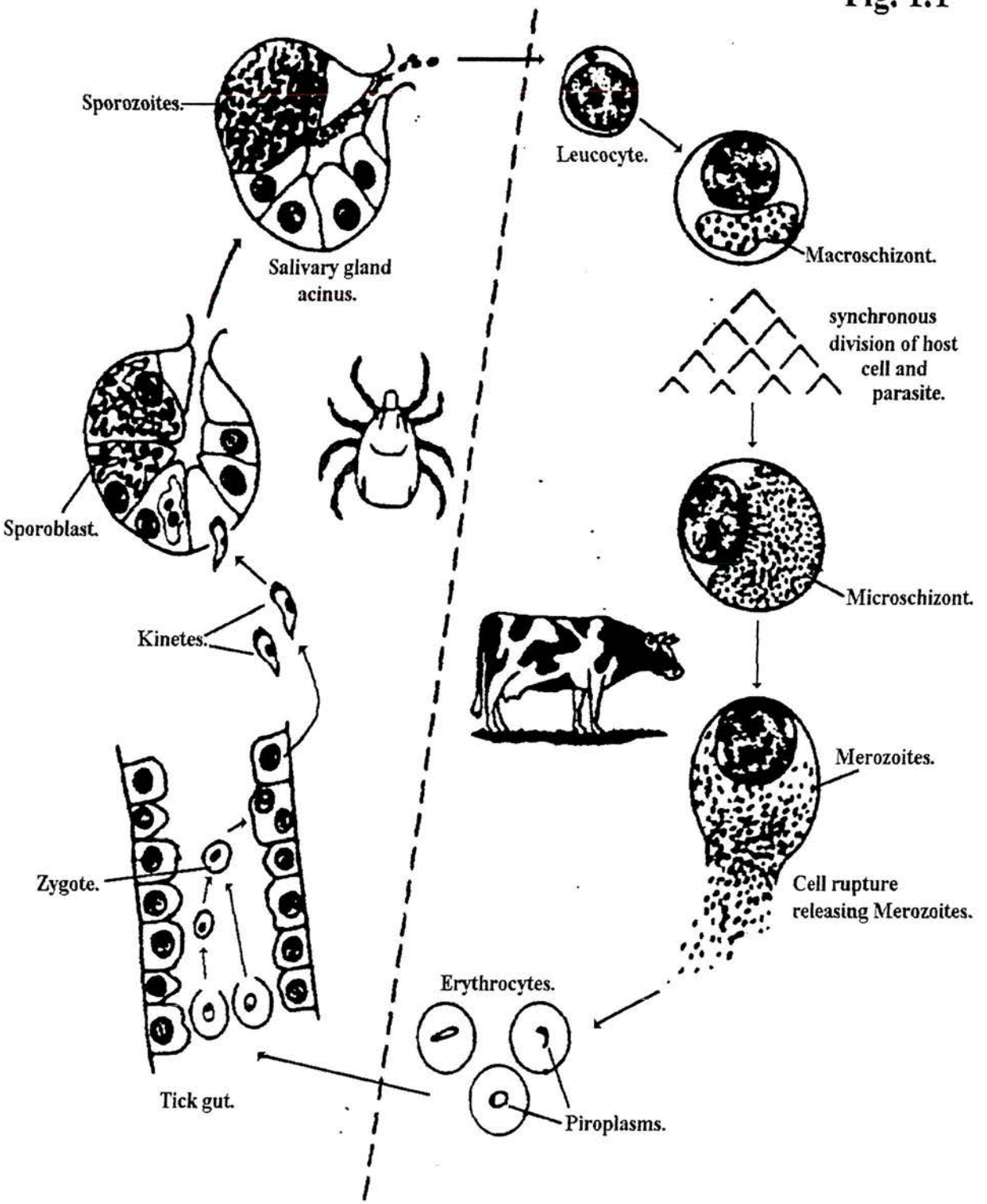
Development within the tick.

The life cycle of *T.annulata* is shown in Fig 1.1. Ticks become infected when they feed upon an infected animal, ingesting a blood meal consisting of piroplasm infected red blood cells (RBC). Once the RBC pass into the tick's digestive tract, the changes in environment cause them to lyse. This lysis initiates sexual reproduction of the parasite. Schein (1975) showed that gametes were detectable 4 to 6 hours following ingestion and that zygotes became visible within the wall of the gut after 3 days. The zygote then matures to form the kinete; this stage of the parasite is motile and has a characteristic club shape. Following the formation of this stage, the kinete leaves the gut wall and enters the haemolymph, migrating to the salivary glands. The duration of this migration is variable and has been reported to take from 7 to 20 days, which may be reliant on the moult stage of the tick (Schein and Friedhoff, 1978; Reid and Bell, 1984).

Fig. 1.1 **The life cycle of the protozoan parasite *T.annulata*.**

A diagram depicting the life cycle of *T.annulata*, showing the vector stages (within the tick) on the left and the stages within the bovine host on the right. Sporozoites enter the bovine host via a tick bite, infecting and replicating within leucocytes. Later stages of the life cycle (merozoites) infect RBCs and form the piroplasms which later infect feeding *Hyalomma* ticks.

Fig. 1.1



T. annulata life cycle.

(Taken form Nichani, 1994).

Penetration of the kinete into the salivary glands only takes place following moulting of the tick (Melhorn and Schein, 1984). Once in the salivary glands the kinetes invade acinii and replicate (Schein and Friedhoff, 1978), forming large masses of parasite material which can be seen to contain nuclear and mitochondrial material (Reid and Bell, 1984). Replication within the salivary gland is only possible after the tick has commenced feeding (Melhorn and Schein, 1984).

Sporozoite maturation and transmission occurs when an infected tick feeds on a susceptible host (Robinson, 1982). If an infected tick feeds on a non susceptible host the sporozoites are induced to mature but are not passed to the host. This phenomenon has been used to obtain mature sporozoites for use in *in vitro* infections. Infected ticks of the species *H.a.anatolicum* fed on rabbits, undergo an increase in sporozoite burden which peaks after three days of feeding (Walker and McKellar, 1983). Maturation has also been linked to high temperatures (Samish *et al*, 1977), which has led to speculation that ticks which become infected in areas with high temperatures may be infective much sooner than those carrying *T.annulata* in cooler climates.

Transmission of *T.annulata* is transtadial, with the parasite being passed from tick to host following moulting to form the next instar (Robinson, 1982). Robinson suggests that transovarial transmission of *T.annulata* is of little importance. The ticks implicated in this phenomenon (namely *H. scupense* and *H. rufipes*) are one host ticks and therefore not major vectors of *T.annulata.* It would thus seem logical to assume that transovarial transmission is of little relevance to infection in the field.

The act of engorgement by a tick on a susceptible animal stimulates maturation of the sporozoites within the tick and causes disruption of the parasite material within the acinii. The parasite material begins to contain aggregates or particles (Reid and Bell, 1984), with these particles forming the rhoptries characteristic of parasites of the phylum Apicomplexa. As noted previously this does not occur if the infected ticks feed on nonsusceptible hosts, with sporozoite development halting at replication and not forming discreet particles or rhoptries.

The passage of infective sporozoites from vector to host occurs after sporozoite maturation, whilst the tick is feeding. Developed sporozoites pass from salivary acinii during feeding and enter the peripheral circulation of the host. The parasite burden of

ticks has been estimated at an average of 50000 sporozoites per acinus. One tick can have a number of infected acinii, meaning that ticks provide an extremely large reservoir of parasite material (Melhorn and Schein, 1984).

Development within cells of the vertebrate host.

After subcutaneous inoculation by infected ticks and passage into the lymphatic system, sporozoites come into contact with host cells. *In vitro* study of the entry of sporozoites into bovine cells has shown that this stage of the parasite first attaches to the host cell. The sporozoite is then able to penetrate the membrane and reside within the host cell cytoplasm as little as 5 to 10 minutes after initial contact (Jura *et al*, 1983; Fawcett *et al*, 1982). When the parasite and the cell come into contact the membrane boundaries appear to merge and the parasite sinks into a depression formed in the host cell. As this depression deepens the host cell membrane begins to cover the parasite. This results in the reformation of the host cell membrane and the presence of the parasite within a vacuole bounded with residual host cell membrane (Melhorn and Schein, 1984).

Sporozoites have been observed to attach to the host cell in various orientations, which do not show the apical complex binding to the host cell membrane. This suggests a mode of entry into cells different from those used by other protozoan parasites such as: *Toxoplasma; Sarcocystis* or *Eimeria spp* (Melhorn and Schein, 1984). Once within the cytoplasm of the host cell, the outer layer of residual host cell cytoplasmic membrane disintegrates and the parasite begins to enlarge. This differs from *Eimeria* and *Plasmodium Spp*, which retain their outer membrane to form a parasitophorous membrane (Melhorn and Schein, 1984).

The invasion and *in vitro* "transformation" of cells after infection with *T.annulata* has been shown to be a temperature dependent process (Hulliger *et al*, 1964; Jura *et al*, 1983) (infected cells are referred to as "transformed", as continuously growing cell lines are formed from infected cells). This involves the invading sporozoites maturing to a single trophozoite, within the cytoplasm of the infected cells, which is followed by division of the parasite material and the formation of a macroschizont.

Parasite replication within the host cell occurs by binary fission, forming large

schizonts containing numerous smaller nuclei (Melhorn and Schein, 1984). Soon after this process begins the host cells are induced to divide. Parasite replication synchronises with that of the host cell and has been shown to occur during the G_2, phase of the cell cycle (Jura *et al*, 1985). During cell division spindle formation occurs within the host cell, with parasite material aligning along these spindles soon after the trophozoite stage (Hulliger *et al*, 1964). During host cell division parasite material moves with the spindles to infect both daughter cells. The division of infected cells has been shown to displace uninfected host lymph node tissue (Melhorn and Schein, 1984) and to cause enlargement of lymphoid tissue (Neitz, 1957; Barnett, 1977).

The replication of *Theileria spp* was studied by Hulliger *et al* (1964) and it was found that parasite replication (but not replication of the infected cells) was resistant to the spindle inhibitor colchicine. Infected cells treated with this drug failed to divide, however, the parasite within these cells continued to divide. This suggests that the mechanics of division, employed by the parasite are separate from those of the cells. However, it would appear that if the infected host cell is prevented from dividing, parasite division is eventually inhibited, as toxic effects (such as foamy changes in cytoplasm and giant cell formation) in the infected cells are observed. This is seen to occur as the number of parasite particles increases to around 100 per cell (Hulliger *et al*, 1964).

If the infected cells are not chemically treated, they undergo schizogony and continue to divide, producing many infected daughter cells. Infected cells have been shown to begin to produce merozoites by 8-10 days after infection. Merozoites are the stage of the parasite which infect RBCs *in vivo* and later produce piroplasms. The trigger for merozoite formation is not totally understood but work by Sheiles *et al* (1994), shows that temperature increases induce merozoite formation *in vitro*. This is possibly linked with the onset of fever, which is a symptom of infection (Neitz, 1957). Rupture of the infected cell releases the merozoites and these then contact and invade erythrocytes. These merazoites now form piroplasms and can either be ovoid/circular in shape, possessing an area of nuclear material at one pole (giving the appearance of a signet ring to the piroplasm) or comma shaped. Of these two forms it is only the comma shaped forms of the parasite which are able to divide, doing so by binary fission. The

numbers of RBCs which can become infected can be very high, with severe cases showing 90% infection (Melhorn and Schein, 1984). The presence of piroplasms within RBCs leads to their ingestion during tick feeding and the infection of fresh ticks to continue parasite dissemination.

Section 1.4.

The vertebrate host and susceptibility.

Although various different *Theileria spp* are known to infect sheep and goats, the main hosts of *T.annulata* have been shown to be: buffalo, yak, cattle and crosses of these animals (Barnett, 1977; Wenshun, 1994). Cells from other animals such as sheep and goats can be infected by *T.annulata* sporozoites *in vitro* but genuine field cases have not been observed (Steuber *et al*, 1986). Infection of indigenous breeds of cattle or buffalo usually result in mild infections (Hashemi-Fesharki, 1988). It has also been shown that the majority of animals within enzootic areas of Morocco carry antibodies specific for *T.annulata* (Oudich *et al*, 1993) and local breeds were much less likely to exhibit clinical disease than Friesians imported to boost milk yields.

The main economic problems occur when exotic breeds of cattle are introduced into endemic areas to increase the productivity of local stock. The mortality rates shown in the literature vary from account to account, with a review by Robinson (1982) stating up to 90% mortality in exotic breeds and only 5% in local breeds, whereas figures given by Hashemi-Fesharki (1988) show mortality rates of 40-80% in exotic breeds and 10% in indigenous breeds in Iran. There are several factors which may cause these variations, including differences in parasite virulence from area to area, differences in vector life cycles, levels of parasite burden (numbers of infected ticks) and also differences in the resistance of indigenous cattle to *T.annulata* infection. These factors make direct comparisons of infections within different areas difficult. However, it can be seen from the literature that the mortality induced by this protozoan in indigenous breeds of cattle is much lower than those of imported breeds, although infection rates are similar (Oudich *et al*, 1993).

However, losses due to mortality are not the sole problem because animals which do

not die or exhibit severe clinical disease will generally show a decrease in productivity due to *T.annulata* infection. The levels of morbidity induced by infection are more difficult to calculate than the mortality rates, resulting in less reliable data concerning these losses. However, field observations by veterinarians in Morocco suggest significant losses in the productivity of indigenous breeds as well as imported cattle (Flach, unpublished observations).

The mechanism or mechanisms of resistance to *T.annulata* infection exhibited by indigenous breeds is as yet unknown. This is because of difficulties in the study of the genetics of resistance in cattle (due to a lack of suitable markers). However, one study has shown that *Bos taurus* x *Bos indicus* cattle are slightly more resistant to infection than pure *Bos taurus* cattle (Preston *et al*, 1992a). This study does not however, agree with data from India, where the majority of cattle are cross breeds and yet tropical theileriosis is still a major problem (Grewal *et al*, 1991). Another problem which complicates studies into resistance is that the vast majority of indigenous cattle surveyed have generally already been exposed to the parasite (Flach and Ouhelli, 1992), thus making comparisons of resistance difficult.

Section 1.5.

Clinical disease and Pathology.

The pathology in susceptible cattle observed in *T.annulata* infection includes pronounced anaemia (with "thin" blood and pale muscles), lymphoid hyperplasia, haemorrhage of the draining lymph node, enlargement of the liver and spleen, oedema and dyspnoea, necrotic lesions in intestinal tract and anorexia (Neitz, 1957; Barnett, 1977; Samantery *et al*, 1980; Preston *et al*, 1992a).

Most of the pathology and clinical disease caused by *T.annulata* infection is probably due to the schizont stage of the parasite life cycle, during which infected cells undergo rapid proliferation (Barnett, 1977; Melhorn and Schein, 1984; Pipano *et al*, 1971; Hooshmand-Rad, 1976). Also, severe clinical cases can be observed in calves which did not exhibit a piroplasm stage to their parasitaemia (Pipano *et al*, 1971). The onset of clinical symptoms is variable, typically between 9-24 days after

infection (Barnett, 1977) and depends on a number of factors such as tick burden and virulence of the protozoan parasite. However, following even the heaviest of infected tick infestations the onset of clinical disease is not observed before 5 days (Barnett, 1977).

One to two days prior to the onset of fever, swelling of the lymph node draining the site of tick infestation can be seen and lymph node biopsies show small numbers of cells containing a small number of schizonts. Within 2-5 days of the enlargement of the draining lymph node piroplasms can be seen in circulating RBCs. This is also observed when the disease is induced by the experimental inoculation of sporozoites (Nichani, 1994; Campbell, 1995). After the onset of fever, schizonts can be found in the majority of tissues (which show hypertrophy) and also in the spleen and liver (Barnett, 1977). In severe cases the fever (in cattle a temperature of 39.5^0C or above) becomes persistent and usually remains until either death or recovery. In some cases there is a remission in fever around the fifth day post infection, with fever rising once again after this.

During the early stages of the febrile period the animal eats normally but appetite is seen to decrease as fever continues. The animal then begins to lose weight rapidly and also loses coat condition. Around this time the faeces increases in water content and diarrhoea follows, often containing blood and mucus. Animals also often develop a sporadic cough, accompanied with nasal and ocular discharges. During the onset of severe disease, petechial haemorrhages of serous and mucous membranes occur and oedema in the lungs causes respiratory distress (Neitz, 1957).

The presence of the parasite has profound effects on the peripheral blood cell pool. Progressive leucocytosis is a characteristic of *T.annulata* infection, with white blood cell (WBC) counts rising from around the normal 10000 cells/mm^3 to numbers as great as 36000 cells/mm^3 (Barnet, 1977). The RBC counts are also affected as the disease progresses, with counts generally falling severely in the later stages of infection and producing a profound anaemia. These symptoms of increased white cell counts and decreased RBC counts are typical of *T.annulata* infection and are probably the most pronounced differences between this infection and that caused by *T.parva* (which causes WBC numbers to fall and only slight changes in RBC counts, as

reviewed by Losos, Infectious Tropical Diseases of Domestic Animals, Chapter II). It has been suggested that the loss of RBCs during *T.annulata* infection could be due to the destruction of cells when the erythrocytic stage of the life cycle (the piroplasms) cause RBC lysis. However, this phenomenon is now known to be associated with the removal from the circulation of infected RBCs by the spleen and liver, although the mechanism for removal is not entirely understood (Uilenberg, 1981). Changes in RBC numbers may be linked to the presence of inflammatory cytokines, especially Tumour Necrosis Factor α (Bielefeldt-Ohmann *et al*, 1989; Sileghem *et al*, 1994; Morrison *et al*, 1981), during illness and this possibility will be discussed in detail later

The multiple lesions seen in severe cases of tropical theileriosis and the presence of infected cells within many organs may be linked to the ability of infected cells to metastasise. Work by Baylis *et al* (1994) has showed that infected cells are capable of digesting basement membrane material and to metastasise and penetrate through basement membranes. This characteristic may be linked with the expression of metalloprotease enzymes by the infected cells and may account for their ability to infiltrate other tissues (Baylis *et al*, 1994; Forsyth *et al*, 1994).

Section 1.6.

Prevention and treatment of infection.

The control of *T.annulata* infection has involved numerous strategies, such as: use of acaracides to decrease tick numbers, cross breeding of cattle to reduce susceptibility, infection of animals with sporozoites followed by chemotherapeutic treatments, administration of attenuated macroschizont infected cell line vaccines (McHardy *et al*, 1985; Hashemi-Fesharki, 1988; Brown, 1990) Of these approaches the most commonly used is that of the administration of attenuated schizont infected cell line vaccines.

However, all of the methods of control listed have drawbacks associated with their use. Acaracides and prophylactic treatments are expensive to use, with these agents usually being supplied by the developed to the developing world. Cross breeding of animals has failed to produce cattle with the required resistance to *T.annulata* infection, possibly due to the inherent difficulties associated with the study of

resistance in outbred populations). Infection and treatment regimes run the risk of inducing pathology prior to treatment, plus, as mentioned, chemotherapeutic agents are generally expensive.

Attenuation of infected cells is associated with the *in vitro* passage of this protozoan parasite (Hulliger *et al*, 1964; Gill, 1976; Pipano, 1977). Propagation of infected cells *in vitro*, appears to alter the reactions induced between infected cells and the bovid host. If vaccines are passaged through bovid hosts, they retain a higher level of virulence than if simply kept in tissue culture, as shown by the S3 challenge strain used in Iran (Hashemi-Fesharki, 1988). The main problem with the use of attenuated cell line vaccines is the high cost of tissue culture maintenance of infected cell lines. However, these cell line vaccines produce strong immunity, which if boosted by natural infection can protect animals for long periods (Baylis *et al*, 1993). Also once a vaccine is available many cattle can be quickly vaccinated, thus reducing the cost. The mechanism by which the parasite decreases or loses virulence is still unknown and production of a vaccine line of reproducibly low virulence often takes up to two to three years. Attenuation of cell lines may be due to negative selection of virulent cells during *in vitro* culture, as suggested by Hooshmand-Rad and Hashemi-Fersharki (1968). This work demonstrated that virulent lines showed the lowest ability to adapt to *in vitro* culture.

Further investigations into the mechanisms which may be involved in the loss of virulence of *T.annulata* infected cells include, (1) studies of the metalloprotease activity of macroschizont infected cells (Baylis *et al*, 1994), (2) alterations in the frequency of certain parasite associated alleles, suggesting selection of different populations of cells in long term culture (Littlebury *et al*, 1994) and (3) studies of differences in glucose-phosphate-isomerase types and restriction fragments revealed by two parasite specific cDNA probes, between virulent and attenuated lines, plus also the expression of a possible parasite and virulence marker bound by the mAb EU106 (Preston *et al*, 1994, 1996). These and other possible mechanisms of attenuation will be discussed further in later chapters.

Other problems associated with this method of tropical theileriosis control are those of cryostorage and transportation in hot climates. In addition there are the difficulties

of maintaining sterile culture conditions, preserving the purity of the vaccine line and preventing contamination with other pathogens.

However, attenuated cell line vaccine administration remains the favoured procedure for the protection of naive animals from field challenge. Various studies have been carried out to assess the most reliable method of vaccine administration (Gill *et al*, 1980; Ouhelli *et al*, 1989). These studies show that administration of infected cells to naive animals produces solid immunity to challenge with various strains of *T.annulata.* This means that a cell line vaccine can be used to immunise animals over a wide area, even if this area contains a range of parasite strains (Gill *et al*, 1980).

The numbers of infected cells necessary to produce immunity to *T.annulata* can vary greatly (Ouhelli *et al*, 1989) and that vaccination is most efficient when administered as a heterologous cell line (Innes *et al*, 1989). During the study by Ouhelli *et al* (1989), it was found that reactions to vaccine lines, (which in this case were unattenuated), such as induction of fever, did not correlate with the dose of vaccine given. Also some animals which were given only 10^2 infected cells showed more severe symptoms than those given higher doses (Ouhelli *et al*, 1989). However, the generally accepted dose range of vaccine administered today is between $1\text{-}5x10^6$ heterologous infected cells, administered subcutaneously, usually in the shoulder (Ouhelli *et al*, 1989). Innes (1989) showed that the use of a non Major Histocompatibility Complex (MHC) matched cell line to vaccinate animals, led to less severe reactions than when a homologous cell line was used as a vaccine. This may relate to the way in which the host detects and responds to the presence of infected cells expressing autologous MHC molecules.

Possible reasons for these phenomena are: administration of a low dose may not trigger a strong enough anti-parasite immune response quickly enough to prevent parasite cross over and development of parasite within cells of the recipient. Once the parasite crosses over into the cells of it's new host, it may be able to replicate unhindered (due to the lack of an anti-MHC response), producing large numbers of parasitised cells before the immune system begins to react to the presence of parasite infected cells. Similarly, use of an MHC matched line may allow the infected cells to undergo many more rounds of replication with no anti MHC response to remove them

from circulation. Innes *et al* (1989) showed that when an allogeneic cell line infected with *T.annulata* was used to immunise animals, an initial cytotoxic T lymphocyte (CTL) response was directed towards the MHC class I molecules, also that parasite cross over into cells of the recipient was essential for the production of a protective response. But later in infection the CTL response was directed towards the infected recipients cells. It was concluded that the initial anti MHC response aids in the delay of the onset of parasitaemia and allows the host to mount a more effective response once the parasite transfers to the recipient host's own cells. This obviously cannot occur when an autologous vaccine line is used.

Another problem with attenuated cell line vaccines, is that of post vaccinal reactions. These reactions occur in animals around days 15 to 20 post vaccination and are characterised as subacute episodes of tropical theileriosis (slight fever, loss of appetite and lethargy) (Dr R. L. Spooner, pers. comm.). The reason for established vaccine lines causing mild disease are unknown but may result if vaccine lines are not fully attenuated. Despite all these drawbacks, use of attenuated vaccines provides the most reliable, widely available protection against *T.annulata* infection.

Chemotherapeutic treatment, though expensive, does provide a final line of defence against infection with this parasite. A number of drugs have been used to treat tropical theileriosis, with most being quinoid derivatives which act on the respiratory chain of the parasite but not the mammalian respiratory chain. The most successful of the chemotherapeutic agents used are derivatives of the hydroxynapthoquinone compound parvaquone. The two most widely employed agents are Parvaquone (Clexon; Wellcome) and Buparvaquone (marketed as Butalex, Mallinckrodt Veterinary Ltd). In a study of the effectiveness of these two drugs McHardy *et al* (1985) showed buparvaquone to be 20 times more effective at clearing *T.parva* and *T.annulata* infections than was parvaquone. The increase in efficiency of Butalex being due to alkylation of the hydroxynapthoquinone. This process removes the cyclohexyl moiety, which the host can metabolise (through hydroxylation), to remove the drug from circulation. This increases the length of time that the drug stays in the host circulation and therefore increases the exposure of the parasite to this drug.

Treatment with Butalex during a *T.annulata* infection results in strong immunity to the

parasite (Dhar *et al*, 1990), with treated animals resistant to 30 times the LD50 dose of sporozoites (Kumar *et al*, 1990; Samantery *et al*, 1980; Preston *et al*, 1992a). A dose of sporozoites is measured in tick equivalents (TEs), 1TE being equal to the sporozoites contained in the acinii of one engorged tick and producing an approximate LD50 (Samantery *et al*, 1990; Preston *et al*, 1992a). The production of solid immunity post treatment shows that removal of living parasite from an infected host, allows the production a protective anti-parasite immune reaction. The reactions induced are capable of protecting the host from either field infection or experimental infection with either sporozoites or a schizont infected cell line (Samantery *et al*, 1990; Preston *et al*, 1992a). However, Butalex treatment is very expensive when compared to the income of the average third world farmer and infection and treatment may cause significant falls in milk yields (Dr R. L. Spooner, pers. comm.)

Attempts were made to use killed schizont infected cells to immunise animals. However, this method resulted in a lack of protection against challenge with *T.annulata* (Pipano *et al*, 1976; Hashemi-Fesharki, 1988). These findings suggest that the presence of live parasite is necessary to allow the production of immunity. It should also be noted that since a single *T.annulata* stock can immunise against stocks from different areas, the parasite does not appear to utilise high levels of antigenic variation to evade the immune system (as do certain organisms, such as Trypanosomes (Borst, 1982; Donelson and Turner, 1985)). It would seem that *T.annulata* employs an alternative strategy to evade the immune system.

Section 1.7.

Immunity to *T.annulata* infection.

Although susceptible animals usually succumb to *T.annulata* infections it is possible for animals to form a protective immune response against this parasite (Nichani, 1994; Campbell *et al* 1995). It has been known for some time that animals which have recovered from the infection show solid immunity not only to the stock of *T.annulata* they became infected with but also cross immunity with other parasite stocks (Gill *et al*, 1980). This suggests that the molecules which the immune system reacts against during a protective response are expressed by *T.annulata* infected cells regardless of

the origin of the parasite stock.

The mechanisms which act against *T.annulata* infections in an immune animal vary depending upon the stage of the parasite life cycle in question. Immunity against the infective stage of the parasite (the sporozoite) is thought to be reliant upon neutralising antibodies, which prevent attachment of sporozoites to bovine cells and probably lead to opsonisation and clearance from the system (Preston and Brown, 1985). Ahmed *et al* (1988) showed that immune serum could prevent sporozoite entry into leucocytes and induce killing of free merozoites but was unable to show any activity against macroschizont infected cells. Therefore, Ab may play a important role in preventing parasite from re-establishing an infection within an immune animal.

However, further work has shown that immune mechanisms are induced which can act against infected cells. Soon after sporozoite invasion a trophozoite forms within the infected cell and Preston, Brown and Richardson (1992) showed that trophozoite destruction can be mediated by cytokines. This study showed that the presence of a number of cytokines including: TNFα, IFNγ, IFNα, IL-1 and IL-2 to inhibited the *in vitro* development of trophozoite infected cells. However, the presence of TNFα was shown to consistently enhance the proliferation of macroschizont infected cells, suggesting that this cytokine may actually exacerbate established infections. Studies by Nichani in naive animals showed that the presence of high levels IFNγ within infected animals did not clear infection (Nichani, 1994). The presence of these two cytokine is normally associated with the initiation of immune reactions capable of removing intracellular parasites (Howard, 1986; Ghalib *et al*, 1995). One may therefore suggest that the roles of cytokines may differ depending upon the particular stage of the parasite life cycle in question and whether infected animals have already been exposed to *T.annulata.*

The destruction of macroschizont infected cells relies upon cytotoxic T lymphocytes (CTLs) specific for infected cells and the presence of cytostatic MΘs (Preston, Brown and Spooner, 1983; Innes *et al*, 1989a,b). CTL responses against infected cells have been demonstrated in *T.annulata* immune animals (Preston, Brown and Spooner, 1983; Innes *et al*, 1989a,b) and work *in vitro* by Conze *et al*, (1994) showed that purified peptide fractions from *T.annulata* infected cells are recognised by *T.annulata* specific

CTL in association with MHC class I molecules. These data show specific CTLs can be generated against macroschizont infected cells and that such CTLs may provide a mechanism of parasite removal, agreeing with the work by Preston, Brown and Spooner (1983) and with suggestions by Ahmed *et al*, (1988).

T.annulata infection in susceptible animals.

The production of immunity in susceptible animals is reliant upon either the inoculation of animals with attenuated infected cell line vaccines, to provide protection against subsequent infection or by drug treatment of infected animals to clear the infection and allow an anti parasite immune response to be mounted (Ouhelli *et al*, 1989; Dhar *et al*, 1990). However, if a susceptible animal becomes infected with *T.annulata* and is not treated then generally the animal will fail to produce a suitable immune response and will die.

The exact mechanisms which prevent a susceptible animal from producing an effective immune response are unknown. Some reasons which could be responsible for this lack of anti parasite activity are: (1) the uncontrolled, rapid proliferation of infected cells; (2) the properties of infected cells, such as the production of cytokines and (3) the effect which *T.annulata* infected cells have upon autologous naive and memory T cells (as shown *in vitro* by Campbell (1995). In this section development of infected cells and their interaction with the susceptible host's immune system will be discussed.

Infected cell development.

In vitro T.annulata sporozoites have been shown to infect principally cells of the monocyte and macrophage (Mθ) lineage (Glass *et al*, 1989; Campbell *et al*, 1994). Once these cells are transformed by the parasite they begin to proliferate, generating continuously growing cell lines *in vivo* and *in vitro* and causing the leucocytosis characteristic of a *T.annulata* infection (Barnett, 1977; Melhorn and Schein, 1984). Prior to *in vitro* infection bovine monocytes and Mθs exhibited a typically myeloid phenotype (*ie*, lobed nucleus, extensive areas of cytoplasm and expressing cell surface markers specific for myeloid cells such as Fc and lipopolysaccheride receptors (Campbell *et al*, 1994). However, following infection with the parasite the phenotype

of the proliferating cells alters.

Infected cells increase in size and show increased but variable expression of MHC class II (Spooner *et al*, 1988; Glass *et al*, 1990). There are changes in the expression of various surface markers, particularly CD14. This molecule is the lipopolysaccheride (LPS) receptor, which is expressed on monocytes, MΘs and a small number of B cells in humans (reviewed by Ziegler-Heitbrock and Ulevitch, 1993) and appears to show a similar expression in cattle (Campbell *et al*, 1994; Yang *et al*, 1995). After infection, cells lose surface expression of CD14 but attain a phenotype similar in other aspects to mature MΘs, as designated by expression of epitopes bound by the mAbs IL-A24 and IL-A109 (Ellis *et al*, 1987; MacHugh, *et al*, 1990; Campbell *et al*, 1994). Expression of the antigen bound by IL-A24 increases after infection, whilst expression of the epitope bound by IL-A109 decreases post infection. IL-A24 binds an epitope expressed by mature MΘs and associated with antigen presentation, whilst the epitope bound by IL-A109 is thought to be analogous to CD64 (FcγR1), the low affinity Fc receptor, found on small monocytes. The fall in IL-A109 binding and the rise in the binding of IL-A24 suggests maturation of the infected cells (Campbell *et al*, 1994).

The identification of the cells infected *in vivo* has proved difficult, mainly due to the fact that once infected, cells rapidly change the surface expression of numerous markers, masking their original identity. However, comparison of flow cytometric data obtained from analysis of *in vivo* and *in vitro* derived infected lines has shown the majority of *T.annulata* infected cells have a similar phenotype (Campbell, 1995), suggesting that the cells infected *in vivo* and *in vitro* are similar. Also new data produced by Forsyth *et al* (1997) shows *in vivo* infected cells express the CD11b, the C3bi complement receptor and CD2. Providing further evidence that *T.annulata* does infected cells of the myeloid lineage *in vivo*.

Studies by other groups have suggested that T cell lineages can also be infected by *T.annulata* sporozoites. One such group Herrmann *et al*, (1989) discussed the expression IL-2 receptor molecules by *T.annulata* infected cell line. However, reports of these cell lineages becoming infected have been limited and certainly in our hands T cells are not infectable (Spooner *et al*, 1989; Campbell *et al*, 1994).

Herrmans work suggesting that infected cell lines contained infected T cells, was

based upon data concerning the uptake of ^{125}I-IL-2 by infected cells. This methodology relied upon the idea that cells expressing the IL-2 receptor will bind free IL-2 and internalise the receptor/ligand complex, thus identifying *T.annulata* infected cells bearing the IL-2 receptor.

However, there are drawbacks to this hypothesis. *T.annulata* has been shown to infect and "transform" cells of the myeloid lineage with much greater efficiency than those of the T cell lineage. Myeloid cells are known to possess the ability to ingest proteins present outside the cell via pino/endocytosis, one must therefore allow for the possibility that the uptake of ^{125}I-IL-2 was due to non-specific internalisation by infected MΘs. It must also be noted that *in vivo*, no evidence has been found that T cells become infected and although infected cells expressing CD25 (the IL-2R α chain) have been identified, no infected cells have been found to express T cell receptors or CD3 as detected by flow cytometry (Campbell, 1995).

In vivo development.

The effects of the presence of parasite within susceptible hosts can be seen soon after an animal is infected by a tick vector or experimental infection. Inoculation of sporozoites into a susceptible host by either route leads to the enlargement of the draining lymph node within 5-8 days (Barnett, 1977). Although the draining lymph node is known to be the main site of development, until recently the site at which cells become infected was unknown. However, recent afferent and efferent cannulation experiments now suggest that the initial site of infection is the draining lymph node. As infected cells are only found in the efferent and not the afferent lymph of the infected node (Nichani, 1994; Goel, 1996).

Immunohistological staining of infected lymph node sections has shown that the numbers of monocytes and MΘs in the draining lymph node begin to increase after infection (Campbell, 1995). Investigation of the expression of the monocyte/MΘ lineage markers bound by the mAbs IL-A109 (MacHugh *et al*, 1990) and IL-A15 (binding CD11b/CD18) (Splitter and Morrison, 1991) in infected lymph node sections and co-staining with the anti proliferation marker mAb Ki-67, showed cells expressing these monocyte/MΘ markers proliferate and colonise the majority of the lymph node

(Campbell, 1995). Also later in the infection there is an increase in the numbers of IL-A24$^+$ cells (Ellis *et al*, 1987) leaving the lymph node (Nichani, 1994). IL-A109 expression has been associated with immature monocytes, whereas IL-A24 (Ellis *et al*, 1987) has been associated with antigen presenting function (which relates to mature monocytes and MΘs). These data suggest that cells of the myeloid lineage within the lymph node become infected, proliferate and express a more mature phenotype, then begin to migrate from the node (Campbell, 1995). This agrees with data collected from many *in vitro T.annulata* infected cultures, showing established cultures of infected cells are mainly IL-A24 positive (Glass *et al*, 1989; Nichani, 1994; Campbell *et al*, 1994).

In susceptible animals the numbers of infected cells can become very large and the immune system is unable to reduce these numbers. The reasons for this are still not known but recent work by Nichani (1994) and Campbell (1995) has shown infected cells alter the behaviour of both naive and memory T cells *in vivo* and *in vitro*. Research into T cell responses during *T.annulata* infection *in vivo* (Nichani, 1994; Campbell, 1995) and post stimulation with infected cells *in vitro* (Campbell *et al*, 1995; Campbell *et al*, 1997) indicates that naive autologous T cells become activated and consistently show a Th_1 type phenotype. Infected cells therefore possess the ability to "non specifically" activate autologous T cells and once non specifically activated, T cells are unreactive against infected cells (Nichani, 1994; Campbell, 1995). *In vitro* work has also shown the T cell stimulatory ability of infected cells to be contact dependent (Campbell *et al*, 1995). When T cells are incubated in the presence of infected cells but separated by a semipermeable membrane the T cells do not activate. This suggests that there is a molecule or molecules present on the surface of infected cells which induces T cell activation and "non-specific" proliferation.

The molecules involved in reactions inducing T cell proliferation are as yet unknown. It has however, been speculated that changes in the levels of expression of MHC class II molecules on the infected cell surface may be involved in the induction of T cell proliferation (Glass and Spooner, 1990). It was found that infected cells possessed enhanced antigen presenting capabilities (when compared to uninfected cells), stimulated increased T cell proliferation, plus also expressed higher than normal levels

of MHC class II molecules. Glass and Spooner hypothesised that it was the altered expression of these molecules that were in some way involved in the abnormal stimulation of T cell activation/proliferation. It is these abilities of T cell stimulation and variations in the levels of MHC class II expression by infected cells, along with cytokine production, which are investigated in this thesis.

The architecture of infected lymph nodes of susceptible hosts post infection.

After infection with *T.annulata* changes begin to take place within the draining lymph node (Campbell *et al*, 1995). In susceptible animals these changes become marked within 4-7 days of infection, with infected cells visible within the node becoming foci for responding T cells, which are seen to activate within 2-3 days post infection. The rate at which these T cells become activated suggests that this process is in some way different to the normal activation process. Campbell's work showed that *in vitro* proliferating T cells are $CD4^+$ and of both the memory and naive phenotype (Campbell, 1995). The activated cells are only present surrounding proliferating infected cells in the medulla, an area where T cell activation does not normally occur (Bogen *et al*, 1991; Bogen *et al*, 1993; Campbell *et al*, 1995). These activated T cells are then seen in the efferent lymph from day 7 onwards. Nichani (1994) also showed that *in vivo* derived, activated T cells from susceptible infected animals were unresponsive to IL-2 and cytotoxic T lymphocytes were not active against infected cells.

Soon after T cell proliferation begins, the structure of the cortico-medullary region starts to break down and the organisation of germinal centres within the node begins to deteriorate. As these reactions proceed the organisation of the lymph node continues to break down and the node can become necrotic within 9 days of infection (depending upon the severity of infection). The rate of apoptosis in the infected node increases between days 2 to 8, then begins to decrease (Campbell, 1995). It has been suggested that this is due to the death of infected cells. As the population of newly infected cells undergoes rapid proliferation, many of the cells initially infected by sporozoites may not survive and undergo apoptosis (Campbell, 1995; Campbell *et al*, 1995).

The premature activation of T cells within the draining node (Campbell *et al*, 1995) may prevent reactions necessary for the formation of reliable T cell help required to mount specific anti theilerial immune reactions. This may also explain why no one has succeeded in making theileria specific T cell lines from immune animals, when using *T.annulata* infected cells as APCs (Campbell, unpub obs). It has been suggested that the aberrant activation of lymph node T cells is the major cause of the lack of a protective immune response against infected cells and one of the causes of severe clinical disease (Nichani *et al*, 1995; Campbell, 1995). Nichani (1994) found large numbers of $CD2^-$ CTLs present in efferent lymph, suggesting that CTLs produced during a non protective immune responses are incapable of normal immune function. If cytotoxic T cells do form the main mechanism for the removal of macroschizont infected cells, then the alteration of T cell responses may well interfere with the production of parasite infected cell specific CTLs. However, this raises a question, if the presence of the parasite induces a Th_1 type response (which would usually be associated with the production of a cell mediated cytotoxic response), why are CTLs isolated from naive susceptible animals not specific for macroschizont infected cells? The answer may lie in the way in which infected cells trigger T cell activation, activation is rapid, antigen non-specific and occurs in areas of lymphoid tissue not usually associated with T cell activation (Glass and Spooner 1990; Campbell, 1995). I have studied possible mechanisms by which *T.annulata* infected cells may activate autologous T cells. The findings of my work are detailed and discussed within this thesis.

Section 1.8.

The Major Histocompatibility Complex (MHC).

Major Histocompatibility Complex (MHC) class II molecule expression of cells alters post infection with *T.annulata* (Glass & Spooner, 1990). Alterations of the expression of these molecules is thought to be related to non specific T cell activation (Glass & Spooner, 1990). For this reason the MHC will be discussed below. The genetics, proteins and function of the MHC have been an area of intense scientific investigation. The initial work in this field concentrated on the human and mouse MHC and

therefore these are the two systems about which most is known. There is less data relating to the bovine MHC but during the last 5 to 6 years advances have been made in understanding the similarities and differences between this and other species.

The MHC Complex is a cluster of highly polymorphic genes on the short arm of chromosome 23 in cattle (chromosome 6 in humans), which are responsible for many essential immunological functions and code for amongst other products, the MHC class I and II cell surface glycoproteins. In humans these molecules are designated HLA molecules (human leucocyte antigens) and in the bovine system were named BoLA or bovine leucocyte antigens (Spooner *et al*, 1979).

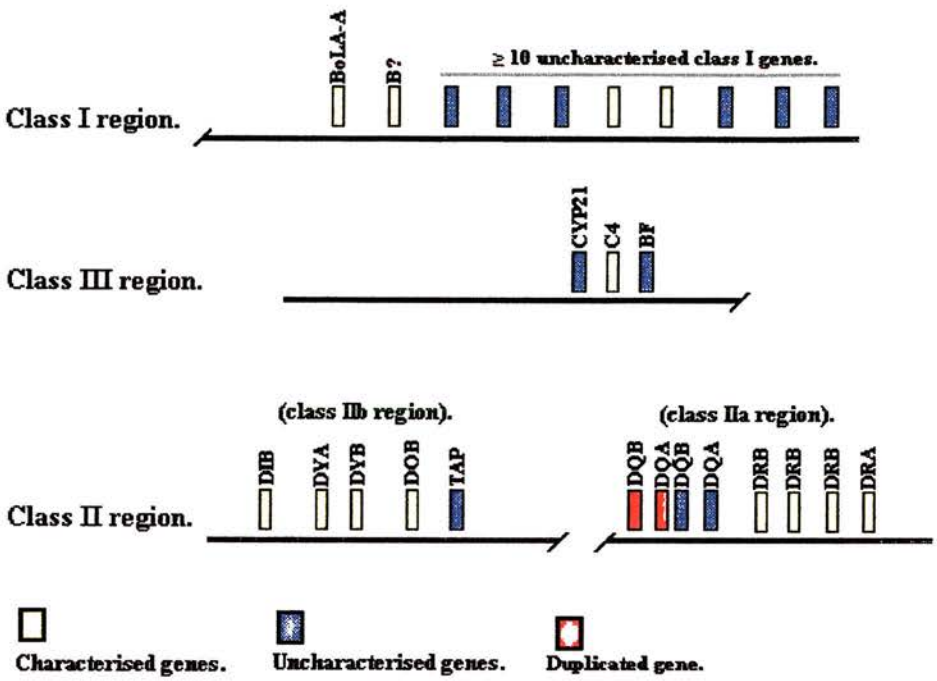
MHC class I and class II products are encoded by different genes and serve different purposes within the immune system. The class I products generally present endogenously derived peptides to cytotoxic T cells and also allow the recognition of self. The class II products mainly present exogenously derived peptides to helper T cells.

The bovine MHC is divided into four areas; class I, class IIa, class IIb and class III (Andersson and Davies, 1993). A map of the bovine map is shown in Fig 1.2 (taken from Sawnhey (1996)). This map is based on data from studies of the bovine MHC, with knowledge of the human, mouse and rat MHCs gene arrangement used to assign likely positions of the bovine genes (virtually no mapping studies of the BoLA system have been performed).

Fig. 1.2 **A map of the bovine Major histocompatibility complex.**

A diagram of the bovine MHC constructed using current knowledge of the bovine MHC genes and also utilising information relating to the organisation of the human MHC. Shown are the class I, class II and class III areas. The class I and II areas contain genes coding for the cell surface MHC class I and class II molecules necessary for the immunological presentation of peptides, whilst the class III region contains genes coding for proteins of the complement system.

Fig 1.2 **Map of the Bovine MHC.**



The BoLA class I loci and products.

There are a large number of BoLA genes (possibly up to twenty) in the class I region (Lindberg and Andersson, 1988). Bovine class I antigens were first described by Spooner *et al* (1978) and Amorena and Stone (1979). These molecules have been identified using alloantisera obtained after immunisation with leucocytes or subcutaneous skin grafting (Spooner *et al*, 1978; Amorena and Stone, 1979). The polyspecific alloantisera produced were tested against a panel of cells and the cross reactivity reduced by absorbtion. This produced (mainly monospecific) alloantisera which could be used in microcytotoxicity assays, for the determination of the MHC class I specificities of particular animals.

The BoLA class II loci and products.

The BoLA class II genes are located in two regions (class IIa and class IIb) and possess a high recombination frequency. The class IIa region is tightly linked to the class I region and contains DQ and DR loci (Andersson and Rask, 1988; Bissumbher *et al*, 1994; Dutia *et al*, 1995). Both genomic and cDNA clones of DR and DQ genes have been isolated and homology with the human genes has been clearly established (Andersson and Rask, 1988). DR and DQ molecules have been identified in the bovine system but no product equivalent to the human DP product has been found (Monaco, 1993; Sigurdardottir *et al*, 1991). Also no evidence for the bovine DP equivalent loci has been reported.

The class IIb region is separated from the rest of the bovine MHC by a recombination frequency of 17% and contains the DOB, DNA, DYA, DYB, DIB and DZ genes (Andersson *et al*, 1988; Stone and Muggli-Cockett, 1990; Andersson and Davies, 1993). The bovine DOB and DNA genes have not been cloned but are assumed to be homologous to their human counterparts and as in the human show only limited polymorphism and do not appear to be expressed (Andersson and Davies, 1993). DYA/DYB are novel bovine genes which also show low polymorphism and do not appear to be expressed (Van der Poel *et al*, 1990). The DIB genes are also thought to be silent.

Studies of the functional bovine MHC class II genes have shown them to be highly

polymorphic (Davies *et al*, 1992), as are their human equivalents, with the potential to present a very wide range of peptides. Comparison of the human DRα and DRβ genes with those expressed by cattle has shown large sequence homologies. Investigation of the DRβ3 genes of cattle showed high levels of polymorphism in exon 2 and this has been identified as the peptide binding region of the class II molecule (van Eijk *et al*, 1992; Russell *et al*, 1994; Fraser *et al*, 1994). The functionally expressed bovine MHC class II molecules identified so far are products of the DQ and DR loci, which present Ag to T cells (Glass *et al*, 1991; Fraser *et al*, 1996).

Antigen (Ag) processing.

Bovine MHC class II molecules consist of a heterodimer of an α and β chain (35 kDa and 28 kDa respectively) (Andersson and Davies, 1993). These protein molecules are synthesised in the rough endoplasmic reticulum (R.E.R.) from messenger RNA (mRNA) encoding the class II genes. In humans the heterodimer associates with another molecule at the E.R, this is the Invariant chain (Ii (CD74)), which can exist in four forms (p33, p35, p41 and p45) (Henne *et al*, 1995). The predominant form of this molecule is p33, with the addition of extra residues on the cytoplasmic tail forming the larger molecules. It is not known if bovids express a functional Ii but since a targeting signal is necessary to allow the class II molecules to reach the endosomes/lysosomes in humans one would assume that this would also be the case for cattle. The exact conformational changes induced by the binding of these molecules are not known (Lamb *et al*, 1992). However, assembly and loading of the class II molecule appears to be less efficient without the presence of the Ii (Sadegh-Nasseri *et al*, 1991; Stern *et al*, 1992).

The presence of the Ii chain does not appear to be essential for class II expression and Ag presentation, as shown by Fraser *et al* (1996). Mouse LTK cells transfected with bovine class II DRα and β genes expressed bovine DR molecules at the cell surface (Fraser *et al*, 1996). Presentation assays showed these cells are capable of presenting Ag to Ag specific bovine T cells. No bovine Ii molecule was transfected with the expressed DR gene suggesting that expression is either possible without such a

molecule or that the mouse cells provide a suitable molecule to aid expression.

The Ii may also be involved in the restriction of class II molecules to the presentation of exogenous peptides (Roche *et al*, 1992. Teyton *et al*, 1990). In humans it appears that antigenic peptides can be bound only when the Ii is removed from the heterodimer. This ensures that binding of Ag is prevented when the class II molecule passes through the Golgi and Trans-Golgi Reticulum (TGR) (which is the area of the cell containing the endogenous Ag pool). Antigen can only bind when the Ii chain is lost, possibly due to a lowering of pH in the MHC containing vesicles (upon fusion with the late endosomes containing the degraded/processed exogenous Ag) (Roche *et al*, 1990). More recent data has suggested that an area of the Ii in humans (termed the CLIP molecule) is able to bind to the peptide binding groove of a large percentage of class II molecules (Ghosh *et al*, 1995). This molecule effectively blocks the peptide binding site until it is removed. Removal of the CLIP molecule is mediated by the human class II molecule DM, which then enables the class II molecule to bind peptide (Sloan *et al*, 1995; Ghosh *et al*, 1995). The presence of bovine DM genes has not been proven but a bovine LMP7 gene has been found. It has been suggested that since the human LMP, TAP and DM genes are adjacent in the HLA system the organisation of these genes will be the same in bovids (Andersson and Davies, 1993).

It is thought that the Ii is also responsible for a sorting signal, which directs the MHC class II heterodimer past the Golgi and TGR. The chains are now directed to the endocytic route and come into contact with internalised and degraded proteins. This sorting signal is thought to be sited in the cytoplasmic tail of the Ii chain, as truncation directs the trimers to the cell surface (Bakke *et al*, 1990, Lamb *et al*, 1991). Further work has suggested that the Ii chains bind with MHC class II heterodimers to form a protein nonamer, consisting of three Ii chains and three MHC class II α/β heterodimers (Cresswell, 1992). The Ii chains contain a retention signal, which is presumed to be situated in the cytoplasmic extension of the p35 form of Ii chain. However, binding of MHC class II heterodimers overrides this signal and initiates transport to the endosomes. This is thought to be due to an endosomal transport signal expressed by the p33 form of Ii. When heterodimers are bound the p35 molecules of the Ii causing retention are covered, whereas the p33 areas are not. This is thought to

bring about transport to the endosomes, where the Ii chains dissociate from the heterodimers which then bind processed peptides (Cresswell, 1992).

The site of intracellular binding of peptide by MHC class II.

The determination of the site of peptide association with class II molecules came closer in 1994 when Tulp *et al* discovered a distinct MHC class II^+ organelle. Fractionation and electrophoretic/immunohistochemical analysis of this compartment showed it to be distinct from lysosomes and early/late endosomes. Within this compartment class II molecules were found to lose the Ii chain (with the NH_2-terminal area of the Ii containing the endosomal targeting signal, being lost last), associate with peptide and become resistant to degradation by SDS (Tulp *et al*, 1994). This data suggests that there are specific compartments for class II loading, which are distinct from compartments used to transport other molecules to the cell surface.

The expression of MHC class II molecules.

MHC class II molecules can be expressed constitutively or have their expression induced. Cells constitutively expressing class II molecules are termed professional APCs and include: dendritic cells; Langerhans' cells; B cells; monocytes and Mθs. Other cell lineages can have class II expression induced by various stimuli, these lineages include: T cells and epithelial/endothelial cells. The stimuli able to induce class II expression include: cell/cell interactions (such as MHC/TCR binding) (Unanue and Allen, 1987); adhesion molecule contact between cells (Larsen *et al*, 1994; Makgoba, Sanders and Shaw, 1989) and cytokines, such as IFNγ (Steeg *et al*, 1982). These factors vary in their action depending on the cell type and conditions involved. For example, IFNγ is one of the most potent inducers of class II expression from monocytes and Mθs and it's effects are exerted through promoters situated upstream of the class II genes (Benoist and Mathis, 1990), *e.g.* expression of the I-Aa gene of mice is effected by TNFα via the NF-κB site (Freund *et al*, 1990), whilst in the HLA-DRA gene the effect is mapped to the S,X and Y regions (no obvious NF-κB site has been found) (Panek *et al*, 1993). One cytokine which is important for the down regulation of MHC class II proteins, especially in professional antigen presenting cells

is Interleukin 10 (IL-10) (de Waal *et al*, 1991; Howard *et al*, 1992). This cytokine appears to play an integral role in the control of inflammatory reactions (Fiorentino *et al*, 1991; de Waal *et al*, 1991). There has been little research into the initiation and control of class II synthesis in bovids, however, one would assume that it will be generally similar to that of the other systems studied.

Antigen interaction with MHC class II products and presentation.

Soon after the biochemical and cellular features of Ag presentation were outlined two different pathways were proposed for the processing and presentation of intra and extracellular Ags. It was suggested that the peptides presented in class I molecules were from cytosolic processing, whilst those presented in class II molecules were derived from endocytic routes (reviewed by Townsend *et al*, 1989 and Chicz *et al*, 1993). MHC class I molecules bind peptides derived from: viruses; intracytosolic bacteria and proteins targeted to the cytosol and endogenously synthesised proteins. In contrast class II molecules bind and present peptides derived from exogenous Ag or Ags within endosomes. These Ags may be derived from soluble proteins or fragments of endocytosed bacteria (Unanue, 1992) and also self peptides (Chicz, 1992). One possible mechanism of dictating whether a peptide is presented by class I or class II molecules is the Ii chain, which prevents the binding of peptides by class II molecules before arrival at the correct compartments (Cresswell, 1992).

For proteins to be presented as peptide fragments by class II molecules, two processes are probably necessary. These are reduction of disulphide bonds to allow unfolding of the protein molecule and enzymic degradation of the unfolded molecule (Allen and Unanue 1984; Collins, Unanue and Harding, 1991). Reduction of disulphide bonds opens up the protein's tertiary structure, revealing concealed epitopes, allowing enzymatic degradation to produce peptides which can then associate with class II molecules.

The point at which molecules enter the endocytic route is still under investigation, but MHC class II molecules have been seen in both early/late endosomes and lysosomes (Harding *et al*, 1991. Peters *et al*, 1991). Studies have indicated that the site of generation of peptides is the lysosome (known for some time to be able to degrade

endocytosed material) (Harding *et al*, 1991). It has been found that targeting of Hen Egg Lysozyme (HEL) to lysosomes results in significantly higher immunogenicity than when targeted to the endosomes (Harding, *et al* 1991). When this is viewed in relation to more recent data, it seems possible that processing may occur within the lysosome but that the processed Ags are moved to the newly discovered cellular compartment to associate with empty class II molecules (Tulp *et al*, 1994).

X-ray crystallographic data of three human MHC class I molecules ((HLA-A2, HLA-A68 and HLA-B27) as reviewed by Germain and Margulies, 1993) allowed the structure and peptide binding functions of this group of molecules to be investigated. However, crystallisation of class II products proved much more difficult, with the crystals formed being too small for analysis. A group eventually crystallised a HLA-DR1 class II molecule from human B cells and its structure was assessed by X-ray crystallography (Brown *et al*, 1993). The structure of this molecule proved remarkably similar to those predicted by theoretical methods and studies of class II function following site directed mutagenesis.

The $\alpha\beta$ heterodimer was found to possess a peptide binding groove between two areas of α helices and the floor of the groove formed from a β-pleated sheet structure. The tertiary structure of the peptide binding groove has also been shown to hold a pocket. The position of this pocket corresponds to the position of a side chain of the bound peptide, with hydrogen bonds formed between the peptide and conserved areas of the peptide binding groove (Brown *et al*, 1993).

The ends of the peptide binding groove were found to be open (unlike the class I peptide binding groove), meaning that the ends of longer peptides are able to droop over the edge of the class II molecule. Brown *et al* (1993) also found that the class II $\alpha\beta$ heterodimers crystallised as dimers. He suggests that this dimerisation may occur at the cell surface and increase the stability of interactions between MHC class II molecules and T cell receptor molecules (TCR). The formation of dimers of class II molecules may also increase their affinity for the CD4 coreceptor molecules expressed on the surface of helper T cells and aggregates of class II/TCRs and CD4 molecules may initiate T cell activation more easily than if single molecules were involved (Brown *et al*, 1993).

The ends of peptides bound by class II molecules are termed as "ragged", since the overall length can vary between 15 and 25 amino acids (Chicz *et al*, 1992). However, the differences in length seem to matter little (with respect to T cell recognition), as long as a certain core motif to the peptide is maintained. Polymorphic variations in the peptide binding groove structure, dictate the peptide motifs which individual class II molecules are able to bind, giving rise to the phenomenon of allele specific peptide binding (Sette *et al*, 1987). The variation in the peptides which a class II molecule can bind is due to the structure and organisation of the MHC binding groove and the motifs of the peptides. The floor of the MHC binding groove contains pockets or depressions, into which fit amino acid (aa) residues (termed anchor residues) of the bound peptide.

However, the binding of ligands by class II molecules does appear to be somewhat promiscuous, with peptides able to bind a number of class II molecules produced from different alleles. The allelic variation of class II molecules means that within a species most animals will express class II molecules able to present the majority of peptides it encounters. Also by the use of tandem mass spectrometry, one species of class II molecule was found to bind 2000 different peptide ligands (Hunt *et al*, 1992). Since as few as 200 MHC molecules with bound peptide are required to trigger a T_h cell (Harding and Unanue 1990), a single APC would be able to present a large number of different peptide epitopes efficiently. These two characteristics allow the MHC class II molecules of a species to present a very large range of peptides, which is very important when one considers the amount of naturally occurring antigenic variation. It would be incorrect to think that the class II molecules only present Ags taken into a cell from the extracellular matrix (exogenous Ags). Class II molecules will present any peptides constructed of a suitable amino acid sequence, present in the novel endosomal compartment at the point of peptide binding. One may think that this would allow the presentation of self peptides and so prompt autoreactivity. However, T cells which would react against these Ags whilst presented by self MHC should have been deleted from the T cell repertoire during T cell ontogeny (Nikolic-Zugic, 1991), thus removing the dangers of mounting an autoimmune reaction.

Also it should be noted that a peptide showing a high affinity for an MHC molecule

may not necessarily prove to be immunogenic. Processing of peptides may produce peptides which bind well to the class II molecule but may not be suitable for mounting an immune response, either because MHC/peptide/T cell reactions fail to form a response or the immune response illicited is not directed at a susceptible area of the pathogen in question.

Section 1.9.

Macrophages, phenotype, functions and intramacrophage parasites.

Macrophages have been known to play an important role in immunological functions for a long time. Possibly the most famous early work on this lineage of cells was that of Metchnikoff . This work consisted of watching cells of the myeloid lineage phagocytose foreign particles and lead to the hypothesis that macrophages played the role of scavengers within the body, mopping up invading organisms and detritus. This is of course true, however, it is now known that monocytes and macrophages play key roles in the activities of many areas of the immune system, including: innate responses (such as inflammation and microbicidal activities); Ag presentation; cytokine production and T and B cell stimulation.

Macrophage phenotypes.

In 1972, cells classified as mononuclear phagocytes were grouped together on the basis of: a common origin; similar morphologies; cytochemistry and function (Van Furth *et al*, 1972). However, following further research it became clear that macrophages found in different tissues possessed a range of varying phenotypes, secretory products and antigen presentation functions (van Furth, 1982; Gordon *et al*, 1995).

Since the initial grouping of mononuclear phagocytes into one group, work in different laboratories has shown that the functions of isolated macrophages vary depending upon the site from which they are isolated but also that macrophages obtained from a single site can also show heterogeneity (Dijkstra *et al*, 1985; Robinson *et al*, 1986; Miller *et al*, 1992). Miller *et al* (1992) showed differential antigen expression between

rat alveolar, pleural and peritoneal macrophages and work by Wewers and Herzyk (1989) demonstrated that freshly isolated human alveolar macrophages activated *in vitro* with LPS release low levels of IL-1β but high levels of TNFα, whilst blood monocytes release higher levels of IL-1β but low levels of TNFα. These data demonstrate the variation in monocyte/MΘ activities and suggest that within the body as a whole these cells play different roles in different cellular and innate immune reactions.

Macrophage Immunobiology.

The immunological activity of MΘs can be split into two areas, that of innate protection and that of acquired immunity. Innate protective mechanisms will be dealt with here first.

Among the innate immunological properties of MΘs are the ability to phagocytose and destroy foreign material (such as extracellular bacteria or protozoa). The degradation of internalised material is achieved by a number of mechanisms, including: protease activity; reactive oxygen intermediate (ROI) synthesis (Nathan *et al*, 1979; Murray and Cohn, 1980) and nitric oxide (NO) activity (Moncada, Palmer and Higgs, 1991). ROIs (especially superoxide) have been known to possess activities against microbial and protozoan pathogens for some time, however in the last few years another anti microbial molecule has come to light. This molecule is nitric oxide, which is produced from L-arginine by nitric oxide synthase (producing L-citrulline as a by-product) (Hibbs *et al*, 1987; Moncada, Palmer and Higgs, 1991). When rats were treated with *E.coli* LPS there was an increase in the urinary output of NO_3^-, which correlated with the levels of fever observed (Wagner *et al*, 1983). Also LPS treatment of LPS sensitive but not LPS resistant mice increased the urinary excretion of NO_3^- and the activation of peritoneal MΘs (Stuehr and Marletta, 1985). These and other data led to the conclusion that it was MΘs which were mainly responsible for the production of NO, which quickly breaks down to form NO_3^-, prior to excretion.

In recent years much research has been carried out into the synthesis and activity of NO, as discussed in a recent review by Liew (1995). Synthesis and release of NO provides an efficient way of dealing with pathogens such as bacteria and protozoa. NO

can act both intra and extracellularly but the range over which the molecule can function extracellularly is limited (probably due to its short half life). The action of NO is thought to be via its effect upon iron-sulphur-centred enzymes within target cells (Pellat *et al*, 1990; Moncada, Palmer and Higgs, 1991) and whether the molecule acts in a cytotoxic or cytostatic way appears to be due to the sensitivity of these enzymes in the various cell types.

There is now evidence for the action of this molecule against numerous organisms including: *Cryptococcus neoformans* (Granger *et al* 1986a, b); *Toxoplasma gondii* (Adams *et al*, 1990); *Leishmania major* (Green *et al*, 1990; Liew *et al*, 1990, *Schistosoma mansoni* (James and Claven, 1989) and *T.annulata* trophozoites (Visser *et al*, 1995). These data show that NO provides a protective and probably very important line of defence against a number of pathogens.

The induction of NO synthesis by macrophages appears to be via two main routes, (1) LPS stimulation and (2) cytokine stimulation (especially IFNγ (reviewed by Green *et al*, 1994)). LPS binds to the LPS receptor (CD14) of monocytes and Mθs in association with LPS binding protein (LBP) (Wright *et al*, 1990), inducing cellular activation and triggering the production of NO. Monocyte and Mθ activating cytokines such as IFNγ bind via their specific receptors to initiate the production of this reactive nitrogen intermediate.

Other innate functions are also mediated through Mθs, the majority of those known appear to be induced and controlled by cytokine molecules released from the activated Mθs. An example of this is the proinflammatory activity of IL-1 (Waage *et al*, 1989). Due to the lack of specific knowledge which exists with respect to the production, structure and activities of bovine cytokines the majority of the following data relates to human or murine cytokines, with the functions of cattle cytokines being extrapolated from this.

Cytokines associated with Mθ function.

Cytokines are pleiotropic molecules which have numerous functions, acting in both innate and acquired immune mechanisms. These molecules are produced by many cells including: monocytes/Mθs; T cells; B cells; NK cells; granulocytes; neutrophils;

basophils; mast cells and epithelial cells. The effects of these molecules are highly specific acting through specific receptors (Stylianou *et al*, 1992). At present there have been 17 interleukin cytokines characterised, with between 2 or 3 identified every year. The MΘ derived cytokines which will be discussed here include: IL-1α; IL-1β; IL-6; IL-10; IL-12 and TNFα (reviewed by Dower *et al*, 1992; Van Snick, 1990; Moore *et al*, 1993; D'Andrea *et al*, 1992; Bacon *et al*, 1995; Beutler and Cerami, 1988). Of these cytokines, IL-1α, IL-1β, IL-6 and TNFα are proinflammatory and have all been shown to play a role in the mediation of endotoxic shock (Waage *et al*, 1989; Creasey *et al*, 1991), producing similar symptoms to those seen during cases of tropical theileriosis.

Interleukin 1α and β.

IL-1α and IL-1β are products of distinct genes, the products of which recognise the same receptors. These two cytokines are structurally related polypeptides showing approximately 25% homology at the aa level (Oppenheim *et al*, 1986). The peptides are produced as 31 kDa precursors and are later cleaved into proteins with molecular weights of approximately 17 kDa (Giri *et al*, 1985; Hazudu *et al*, 1988). There are two receptors for these cytokine molecules but the activities shown to be transduced through each are still under investigation. The type 1 receptor has a cytoplasmic domain of 213 aa residues, whilst the type 2 has only 23 aa cytoplasmic residues (Dower *et al*, 1992). IL-1α has been shown to bind preferentially to the type 1 receptor and IL-1β preferentially to the type 2 receptor (Dinarello, 1991; Dower *et al*, 1992). These data have led to the idea that the type 2 receptor is involved in the regulation of IL-1 by acting as an antagonist or soluble receptor (Sims *et al*, 1993). Glucocorticoids have been shown to up regulate the type 2 receptor, possibly explaining some of the anti-inflammatory effects of these compounds (Colotta *et al*, 1993). The results of IL-1 production upon cells have been shown to be: induction of prostaglandin (PGE_2) synthesis by endothelial cells and smooth muscle; stimulation of the acute phase response by liver and an increase in glucogenesis; collagen production by fibroblasts; the induction of slow wave sleep via action of the central nervous system and stimulation of monocytes/MΘs to produce IL-1/IL-6 and TNFα. IL-1 also has effects in acquired immunity *ie*, stimulation of B cells to increase Ig

production and also the activation of T cells leading to production of IL-2/IL-2R/IL-4 and granulocyte/macrophage colony stimulating factor (GM-CSF) (Unanue *et al*, 1984).

Interleukin 6.

IL-6 has been shown to be produced by numerous cell types including; T cells; B cells; monocytes/Mθs; fibroblasts; hepatocytes and vascular endothelial cells. Its expression has been shown to be upregulated by IL-1, IL-2, TNFα and IFNγ (reviewed by Van Snick, 1990). Human and murine IL-6 are 212 and 211 aa in length respectively, containing hydrophobic signal peptides of 28 and 24 aa respectively (Van Snick, 1990; Akira *et al*, 1993). This cytokine exerts its effects through an 80 kDa binding protein and a 130 kDa signal transducing protein (Hibi *et al*, 1990). As with IL-1 the functions of IL-6 are numerous and are induced in many cell types. IL-6 shows effects on: B cell differentiation and Ab production; induction of the IL-2Rα chain and T cell differentiation/activation; haemopoietic progenitors in synergy with IL-3 to decrease G_0 time; the liver inducing acute phase responses (Ogawa, 1992).

Tumour necrosis factor alpha (TNFα).

TNFα (also known as cachectin) is another of the proinflammatory cytokines produced by monocyte/Mθs (Beutler *et al*, 1985). This cytokine has a relative molecular mass of 17.35kDa (Jones, Stuart and Walker, 1989) and possesses a trimeric tertiary structure in its active form. It also shares 30% aa homology with another molecule called TNFβ (also known as lymphotoxin). However, the activities of these two molecules are markedly different. TNFα will be dealt with here, as it is this cytokine which possesses proinflammatory activity and is produced by Mθs. Mature human TNFα is a polypeptide of 157 aa (with mouse, rabbit and rat being one aa shorter), with an apparent molecular weight of 17 kDa (Vilcek and Lee, 1991).

There are two main TNF receptors, termed TNFR-I (55 kDa) and TNFR-II (75 kDa) as well as a number of smaller soluble receptors. The binding of the TNF molecules to their receptors is an interesting area of investigation, with signalling probably occurring when two or three identical subunits are brought together, through binding

a TNF molecule. The binding and signalling is thought to be similar for both TNFα/β but has only been demonstrated for TNFβ (Jones *et al*, 1989; Eck *et al*, 1992). There has also been a ligand passing model put forward for TNF binding (Tartaglia, Pennica and Goeddel, 1993). This model suggests that TNFα binds to and localises around the TNF-R2 receptor and this regulates the association to the TNF-R1 receptor (which transduces a signal into the cell) by rapid association/dissociation at the cell surface. There is now evidence that the 55 kDa receptor is associated with the lytic activity of TNF binding, whilst the 75 kDa receptor modulates proliferative and regulatory signals in lymphocytes. However, more work is necessary in this area (Tartaglia *et al*, 1993a, 1993b). TNFα is also involved with acquired immune reactions and possesses the ability to activate T cells (Reem *et al*, 1989; Tartaglia *et al*, 1993b). This work showed that TNFα could activate and stimulate the proliferation of T cells and that this occurs via binding to the TNF-R2 receptor.

TNFα is a potent activator of inflammatory responses, mainly due to its activating effect on monocytes and Mθs (Epstein, 1986; Bachwich *et al*, 1986). The activation of these myeloid cells induces the synthesis and release of both IL-1 and IL-6. This leads to an increase in inflammatory reactions and the recruitment of cells such NK cells and neutrophils to the site of inflammation (Tracey, Lowry and Cerami, 1987; Beutler and Cerami, 1987). The ability to induce these reactions means that TNFα plays a large and important role in immune responses to remove pathogens. However, this cytokine has also been implicated as a cause of pathology when present at high levels or for prolonged periods (Tracey *et al*, 1988; Bielefeldt-Ohmann *et al*, 1989). Septic shock is a condition induced by the presence of lippopolysaccheride (LPS) from gram negative bacteria. This molecule is able to bind to the LPS receptor present on monocytes/Mθs and some B cells (Raetz *et al*, 1991; Ziegler-Heitbrock and Ulevitch, 1993). The binding of this receptor by LPS stimulates these cells to produce IL-1, IL-6 and TNFα. These cytokines (especially TNFα) then produce cytokine and inflammatory cascades, resulting in the systemic upregulation of prostaglandins and the induction of fever (Dayer *et al*, 1985). Research in a number of systems has shown that the presence of high levels of TNFα alone are able to induce many of the symptoms observed in septic shock (Tracey *et al*, 1987; Gaskill, 1988; Bielefeldt-

Ohmann *et al*, 1989).

Bielefeldt-Ohmann *et al* (1989) showed the chronic symptoms induced by recombinant TNFα in cattle to include: fever; weight loss (cachexia); depression; anorexia; diarrhoea and tissue damage to the kidneys/liver and lymphoid organs. This work demonstrates the necessity of tight control over the production/secretion of cytokines and is possibly of relevance to research into tropical theileriosis, as these are similar symptoms observed following infection with *T.annulata* (Neitz, 1957; Barnett, 1977).

Macrophages and acquired immune reactions.

Macrophages also play a role in acquired immunity as well as innate protection. Probably the most important role of Mθs (apart from the innate immune functions they perform) is that of providing stimuli for T and B cells during cell mediated immune reactions. Mθs constitutively express MHC class II antigens and are professional Ag presenting cells. This means that Mθs are able (once they have phagocytosed the material in question) to degrade and process molecules and present them in the context of MHC class II molecules to $CD4^+$ T helper cells, allowing the formation of Ag specific immune reactions.

The induction of immune responses by MHC class II/peptide/TCR interactions.

Antigen specific activation of T cells.

The reactions mediated by the bovine MHC class I and class II molecules have been shown to be restricted to appropriate T cells, as in the human and murine systems. Bovine class I molecules act as restriction elements for $CD8^+$ T cells (Eugui and Emery, 1981; Goddeeris *et al*, 1986; Goddeeris, Morrison and Teale, 1992; Innes *et al*, 1989a,b), whilst the class II molecules act as restriction elements for $CD4^+$ T cells (Rothel, Dufty and Wood, 1990; Glass *et al*, 1991)

For correct signalling via the MHC/TCR complex it is also necessary for MHC molecules to contact the CD3 and CD4 molecules expressed by the T cell (as reviewed by Finkel, Kubo and Cambier, 1991). These cognate interactions, together with several non-antigen specific interactions including accessory molecule interactions

(such as; B7/CD28 (Larsen *et al*, 1994; Stein *et al*, 1994) and ICAM-1/LFA-1 (Van Seventer *et al*, 1990)) and cytokine interactions (such as the effect of IL-1 on T cells (Kaye and Janeway, 1984), lead to T cell activation via intracellular second messenger signals. The networks of intracellular second messenger molecules are highly complex and remain an area of intense research.

Following activation via normal MHC/peptide/TCR reactions numerous changes occur within the naive T cell which include the upregulation of certain surface molecules (*eg* class II) and the secretion of cytokines such as IL-2 and IFN-γ (reviewed by Pichler and Wyss-Coray, 1994). T cell stimulatory cytokines (*eg*, IL-2) secreted by the responding $CD4^+$ T cells stimulate clonal proliferation of memory/primed T cells, producing large numbers of antigen specific T cells (also Ag-specific memory T cells). The activated T cells are then able to activate effector cells. These effector cells include a wide range of cell types (ie, monocytes/MΘs, granulocytes, natural killer (NK) cells, neutrophils, cytotoxic T cells, B cells and vascular epithelium). Once activated these cells act against the organisms or material eliciting the reaction. Therefore, the main response mechanism of helper T cells is to secrete cytokines which act on potential effector cells, these cells producing reactions directed against the invading organism (King *et al*, 1990; Miller, 1990; Grau *et al*, 1991).

Non classical T cell activation.

As noted a characteristic of *T.annulata* infected cells is the ability to non-specifically activate autologous T cells. This can also occur when T cells are exposed to mitogens (such as concanavalin A and phytohaemagglutinin) or superantigens (such as toxins produced by Staphylococcal bacteria *ie*, Staphylococcal enterotoxin B (SEB)) (reviewed by Webb & Gascoigne, 1994). These molecules activate T cells by stimulating the cells through non polymorphic regions of the TCR (Roitt, 1988). Mitogens are generally lectin molecules which bind glycoproteins and interfere with the normal signalling between the MHC and TCR molecules. These reactions induce second messenger cascades within T cells and initiate non antigenically restricted reactions.

Superantigens bind to the lateral faces of the MHC and TCR molecules, bypassing the

normal site of Ag presentation (reviewed by Webb and Gascoigne, 1994; Acharya *et al*, 1994). The binding of a particular superantigen to the lateral side of the TCR relies on the Vβ expression of the T cell. The expression of Vβ genes being an essential part of TCR expression, with the products of these genes forming the less polymorphic β strands of the TCR. Activation of the T cells therefore occurs in a very Vβ specific manner, with the stimulation and expansion of only those cells with the correct Vβ structure to bind the superantigen.

The reactions induced by this non-antigen specific activation can be catastrophic, inducing shock and sometimes death *eg*, toxic shock syndrome, caused by SEB (also known as toxic shock syndrome toxin 1 or TSST-1) (Swaminathan *et al*, 1992). The effect of stimulation by superantigens upon second messenger systems is still under investigation. However, following stimulation by a superantigen T cell clones have been shown to proliferate but not to show the expected rises in intracellular second messengers (such as inositol phosphates or Ca^{++} fluxes (O'Rourke *et al*, 1990; Liu *et al*, 1991; Gaugler *et al*, 1991).

Many of the deleterious results of these stimulations are mediated through cytokine release, after the activation of T cells (Kappler *et al*, 1992). The activation of T cells by autologous APCs infected with *T.annulata* shows aspects akin to those of T cell activation by bacterial superantigens. These similarities will be discussed in the following chapters.

T cell responses and T helper subsets.

Research into T cell responses has shown that in certain systems, murine helper T cells can be placed into phenotypic groups based on their cytokine profiles (Mosmann *et al*, 1986). The work in humans showed that $CD4^+$ helper T cells could be placed in three subtypes, T helper 1, 2 or 0 (Th_1, Th_2 and Th_0) (Elson, *et al*, 1995). The functions of these differing subtypes is to mediate different aspects of the immune system's responses. Th_1 cells have been associated with the initiation and control of cell mediated responses via the production of cytokines IL-2, IL-12 and IFNγ, with these cells involved in the stimulation of delayed type hypersensitivity reactions (DTH) and cytotoxic T cells production/stimulation. Th_2 cells show a cytokine profile

which is primarily involved in the control of humoral responses. Two important cytokines involved in the mediation of these reactions are IL-4 and IL-10 (Mosmann *et al*, 1986; Cher and Mosmann, 1987; Scott *et al*, 1987; Paul and Ohara, 1987; Hsieh *et al*, 1992).

The third set of helper T cells has been termed Th_0, which were first thought to be simply a precursor phenotype for the $Th_{1,2}$ cells. However, data from humans and mice suggests that $Th_{1,2,0}$ are produced from the same progenitor pool and the development of the subsets depends upon the stimuli which the T cells experience (such as Ag type and cytokines in their microenvironment) (Rocken *et al*, 1992; Del Prete, Maggi and Romagnani, 1994; Sad and Mosmann, 1994). Work in cattle agrees with these findings and showed that some Th_0 cells are able to produce not only IL-2 and IFNγ but also IL-4 (Brown *et al*, 1993; 1994). These cells were also shown to be specific for Ags of *Babesia bovis*, suggesting that what were thought to be intermediates in T cell development are actually functional T cells.

The exact mechanisms by which T cells diverge into subsets are not known, however, Figures 1.3 & 1.4 (taken from Del Prete, Maggi and Romagnani, 1994) show possible mechanisms for the production of polarised T cell subgroups. These diagrams are based on the human system (however, due to the similarities between the bovine and human T cell subdivisions these models are probably also relevant to the bovine system). Fig 1.3 represents a possible mechanism for the production of a Th_1 response. This mechanism relies on the APC producing IL-12 and stimulating the production of IFNγ by activated NK cells. The presence of high levels of IL-12 and IFNγ suppress the production of a Th_2 reaction, causing the Th precursor (Th_p) cells to produce a Th_1 response (with the synthesis of IL-2 and IFNγ). This sequence of events leads to the activation of B cells and MΘs and the initiation of cell mediated immune responses (Nathan *et al*, 1983; Mosmann and Coffman, 1989; Del Prete, Maggi and Romagnani, 1994).

Fig. 1.4 outlines a mechanism by which Th_2 cell responses could be initiated. This process involves the production of more IL-4 than IFNγ or IL-12. The source of the high IL-4 levels is not absolutely certain, Del Prete (1994) suggesting that mast cells or basophils may supplement the IL-4 produced by the $Th_{0,2}$ cells. Following initiation

of these reactions the high IL-4 and low IFNγ maintain Th_2 development. These reactions result in stimulation of B cells (via cell contact and IL-4/IL-6 production (reviewed by Paul, 1991; Van Snick, 1990; Del Prete, Maggi and Romagnani, 1994)). Th_2 cells also aid antibody (Ab) production (also isotype switching) and the down regulation of inflammatory responses (Alderson *et al*, 1987; Hudak *et al*, 1987; Paul *et al*, 1991).

Fig. 1.3 **A schematic representation of a possible mechanism for the preferential development of a Th1 response following stimuli from intracellular bacteria and viruses.**

A diagram outlining a possible mechanism by which an animal may mount a T helper type 1 response acting against an infection with intracellular bacteria or viruses. Endogenous antigens arising from pathogens are processed and presented in the context of MHC class I molecules to T helper cells. The cells presenting the antigens also provide soluble cytokine messengers (*i.e.* IL-12 and IFNα) which promote the T helper cells to initiate a type 1 response (also mediated by soluble factors, including IL-2 and IFNγ), involving macrophage activation and delayed type hypersensitivity reactions.

Fig. 1.3

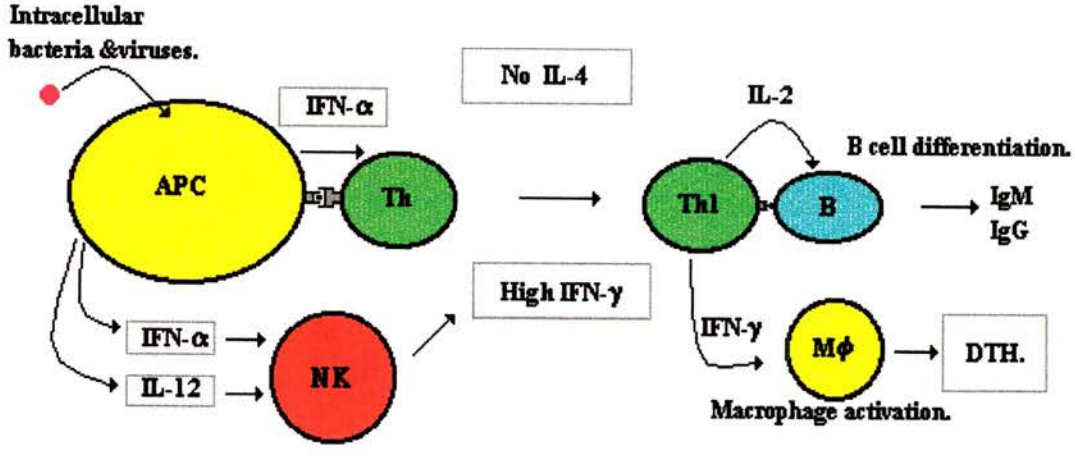
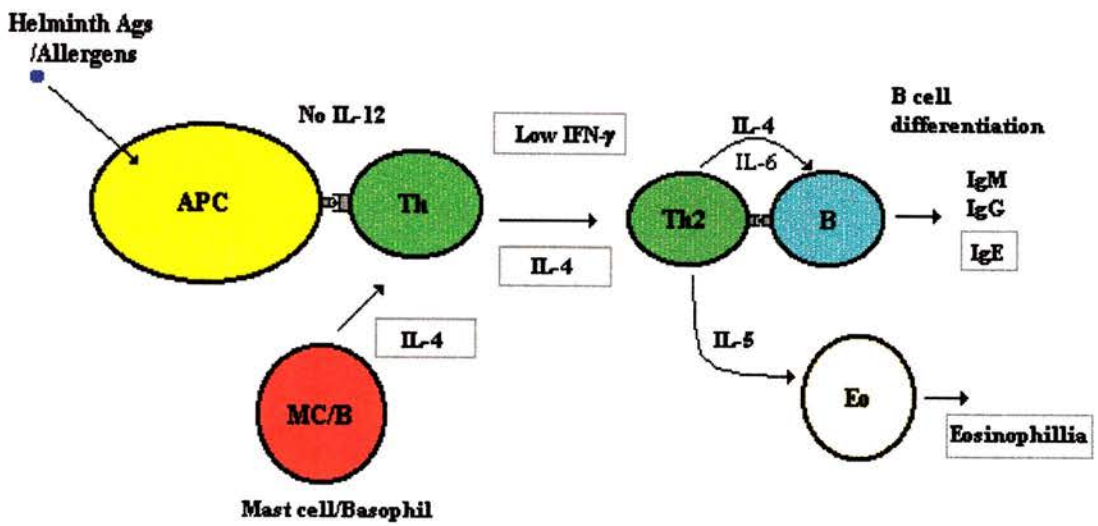

Intracellular
bacteria &viruses.
APC
IFN-α
Th
No IL-4
IL-2
B cell differentiation.
Th1
B
IgM
IgG
IFN-α
IL-12
NK
High IFN-γ
IFN-γ
Mϕ
DTH.
Macrophage activation.

Fig. 1.4 **A schematic representation of a possible mechanism for the preferential development of a Th2 response following stimuli from allergens and exogenous materials.**

A diagram outlining a possible mechanism by which an animal may mount a T helper type 2 response acting against an infection with extracellular pathogens. Exogenous antigens arising from pathogens are endocytosed and processed/presented in the context of MHC class II molecules to T helper cells. The cells presenting the antigens also provide soluble cytokine messengers (*i.e.* IL-4) which promotes T helper cells to initiate a type 2 response (also mediated by soluble factors, including (IL-3, IL-5 and IL-6), involving B cell activation/differentiation, Ab production (of Igm, IgG and IgE), as well as eosinophil activation.

Fig. 1.4


Helminth Ags
/Allergens
No IL-12
APC
Th
Low IFN-γ
IL-4
IL-4
IL-6
B cell
differentiation
Th2
B
IgM
IgG
IgE
IL-4
MC/B
Mast cell/Basophil
IL-5
Eo
Eosinophillia

The actions of these two groups of cytokines are also antagonistic with for instance, Th_1 cytokines upregulating MΘ activity, whilst Th_2 cytokines downregulate this action and suppress inflammatory reactions. The segregation of these responses means that the stimulation and expansion of a particular T cell subset determines the response against a particular pathogen. The most suitable T cell response varies depending on the nature of the pathogen/infection involved. This can be seen in the responses to infection with intracellular parasites of the genus *Leishmania*. It has been shown for both *L.donovani* and *L.major* that Th_1 mediated responses are able to produce anti-leishmanial activity, with special emphasis placed on the role of IL-12 and IFNγ, which act as powerful stimuli for MΘ activation (Wang *et al*, 1994; Nathan *et al*, 1993; Ghalib *et al*, 1995). IFNγ has also been shown to induce killing of *Toxoplasma gondii* infected cells, by activating MΘs (Suzuki *et al*, 1988). With these facts in mind, one would think that the presence of IFNγ would be beneficial for the host during *T.annulata* infection (*ie*, that IFNγ would activate MΘs and induce killing of intramacrophage *T.annulata*). However, data produced by Nichani (1994) and Campbell *et al* (1997), showed very high levels of IFNγ during *T.annulata* infections, which appeared to have little effect upon parasite burden.

However, the placement of T cells into these rigid subgroups maybe an oversimplification of the true facts. This is possibly due to the methods by which the majority of this data has been produced. The majority of studies relying upon the generation and *in vitro* study of T cell clones. Many of the systems used to study the phenomenon of T cell subsets have relied on the cloning and *in vitro* stimulation of T cells with mitogens or high levels of cytokines (Firestein *et al*, 1989; Street *et al*, 1990). These are artificial systems and one wonders about the validity of using these systems to describe the activities of normal non clonal T cell cultures.

CD8$^+$ cytotoxic cells.

An important mechanism of adaptive immunity is the production and expansion of CD8$^+$ cytotoxic T cell populations, which are able to destroy infected cells. Investigations into the role of these cells during *T.parva* infections showed that transfer of *T.parva* specific CTLs protected recipient cattle from a lethal sporozoite

challenge (McKeever *et al*, 94). It has also been suggested that these cells also play a role in the removal of *T.annulata* macroschizont infected cells (Preston *et al*, 1983; Innes *et al*, 1989a,b). The activity of cytotoxic $CD8^+$ T cells is in part mediated by $CD4^+$ T cell subsets and stimulation/activation of cytotoxic T cells can result in the removal of intracellularly infected cells (*ie*, infected with viruses, bacteria or protozoa). Bovine CD8 is an antigen consisting of an alpha chain (34 kDa) and beta chain (38 kDa) and is found on T lymphocytes which mediate cytotoxicity through recognition of Ags presented in the context of MHC class I molecules (MacHugh and Sopp, 1991). The specificity of these cytotoxic cells is controlled via TCRs specific for peptide epitopes expressed in the context of MHC class I.

Binding of this epitope/class I complex, as well as costimulatory signals such as the presence of cytokines (*ie*, IL-2/IFNγ) activates the $CD8^+$ cell whilst in close proximity to the infected cell. Once this mechanism has been set in motion the cytotoxic cell secretes degradative lytic granules, which perforate the target cell's membrane, killing the cell (Poenie *et al*, 1987, Takayama *et al*, 1987). A molecule linked with the toxicity of these lytic granules is perforin, which has been shown to damage cell membranes (Kagi *et al*, 1994). Cytotoxic cells can also induce the expression of the *Fas* molecule which transduces an apoptosis signal into the infected cells, which also causes cell death (Kagi *et al*, 1994). Once damaged the cells loose integrity and are phagocytosed by cells such as MΘs and other mononuclear phagocytes.

Cytokines affecting T cell responses.

The four main T cell derived cytokines dealt with in this work are: IL-2; IL-4; IL-12 and IFNγ. Also addressed is the production and activity of another cytokine produced by T cells, IL-10. These cytokines are produced by T cells, however, other lineages can also make some of these molecules. For example, IL-4 can also be produced by Mast cells, IL-10 by monocytes and MΘs and IFNγ by NK cells. These four cytokines play very important roles in the induction and maintenance of acquired immune reactions and the regulation of MΘ activity (as reviewed by Doherty, 1995).

Interleukin 2.

IL-2 is a cytokine produced by activated T cells which stimulates T cell responses

(Morgan *et al*, 1976). This cytokine aids the activation of T cells and later in the clonal expansion of Ag specific T cells. Human IL-2 is a polypeptide of between 15 and 18 kDa. Following synthesis the peptide is 153 aa in length but 20 of these aa constitute the signal sequence (which is later cleaved leaving the mature 133 aa factor). In humans the IL-2 receptor consists of three glycoprotein chains. IL-2Rα shows low affinity for IL-2, IL-2Rβ contains the cytoplasmic domain used to transduce the IL-2 signal and the IL-2Rγ chain associates with these to allow high affinity binding of IL-2, ligand internalisation and signalling (Hatakeyama & Taniguchi, 1990; Smith, 1989; Takeshita *et al*, 1992). However, in cattle this receptor only appears to consist of two peptide chains α and β but nevertheless shows a high affinity for IL-2 (Siess and Reeves, 1989).

Interleukin 4.

IL-4 is also produced by activated T lymphocytes but the production of this cytokine depends upon the particular subset of T helper activated. This phenomenon of T helper cell subsets producing differing cytokine profiles has undergone intense investigation and will be discussed in depth in a later section. IL-4 has been shown to be an extremely pleiotropic cytokine, which has effects upon amongst others: T cells; monocytes; Mθs; mast cells; fibroblasts; endothelial cells; and hepatocytes. This cytokine is produced by $CD4^+$ T cells as well as mast cells, basophils and $CD8^+$ T cells. Human and mouse IL-4 are 129 aa and 120 aa long respectively. Bovine IL-4 has recently been cloned and sequenced, the sequence codes for an initial product of 15.1 kDa, 135 aa peptide with a putative 24 aa leader sequence and a 121 aa product (Heussler, Eichhorn and Dobbelaere, 1992).

IL-4 activity is mediated by receptor complexes, of which there appear to be at least two forms. Most information is known about the IL-4 receptor which consists of an IL-4 binding subunit (IL-4R) and a γ chain (this γ has been shown to be the same chain which acts to bind IL-2 at high affinity in this receptor complex) (Russell *et al*, 1993; Kawahara *et al*, 1994). This extra chain appears (as for IL-2 to the IL-2R complex) complex to facilitate high affinity binding of IL-4 (Russell *et al*, 1993). However, there also appears to be an alternative IL-4 receptor complex, as

demonstrated by Lin (1995), who showed that some cells which did not express the common γ chain were still able to transduce IL-4 signals (Lin *et al*, 1995).

IL-4 has been shown to play an important role in the differentiation of T helper cells to a Th_1 or Th_2 like phenotype (Del Prete, Maggi and Romagnani, 1994) and to down regulate the activities of Th_1 like cells, such as IFNγ production (Paul *et al*, 1991). Principle upregulatory activities of IL-4 are exerted upon B cells: causing increases in cell viability and size; upregulation of surface molecules (*ie*, CD23 (low affinity Fc receptor), MHC class II, IgM and B7) and influencing Ab switching (Paul *et al*, 1991; Gollob and Coffman, 1994). Another very important activity of IL-4 is the downregulation of inflammatory responses, with this cytokine inhibiting proinflammatory cytokine (IL-1, IL-6 and TNFα) release from MΘs and causing the down regulation of CD14 expression by these cells (Lauener *et al*, 1990). Work on bovine recombinant IL-4 by Estes *et al* (1995), has confirmed that this cytokine plays an important role in T and B cell reactions (as in other systems) and also that the presence of IL-4 increased B cell proliferation in mixed PBM cultures and upregulated the surface expression of CD23, MHC class II and IgM (as is observed in the human and murine systems). However, this work did show that the effect of bovine IL-4 upon Th_1 and Th_2 clones differed from that seen in the murine system, with this cytokine inhibiting the proliferation of both subsets in a dose dependant manner (Estes *et al*, 1995).

Interferon gamma.

Interferon gamma (IFNγ) is the product of a single gene (Gray *et al*, 1982) and is a 143 aa glycoprotein in humans, the relative molecular mass of which depends upon the glycosylation state of the peptide (Rinderknecht, O'Connor and Rodriguez, 1984). The 25 kDa peptide has two glycosylated sites, whilst the 20 kDa peptide is only glycosylated at one site. However, glycosylation at neither site is essential for biological activity. The receptor for IFNγ is highly species specific and is also the product of a single gene (Auget *et al*, 1988).

This cytokine is produced by: Th_1 $CD4^+$ T cells; $CD8^+$ T cells and NK cells and possesses antiviral and antiprotozoal activities (Fleischmann *et al*, 1979; Suzuki *et al*,

1988). However, bovine γδ T cells do not produce IFNγ (Collins *et al*, 1993). IFNγ is a potent activator of monocytes and Mθs, with the presence of this molecule upregulating MHC class I and II expression, IL-1 and superoxide production and in the presence of LPS induces large scale NO synthesis (Bielefeldt-Ohmann *et al*, 1986; Nathan *et al*, 1983; Gordon *et al*, 1995). Ab production is also enhanced by IFNγ, with IgG_{2a} synthesis being upregulated in B cells exposed to LPS and IFNγ (Snapper *et al*, 1988). IFNγ can also induce the upregulation of ICAM-1 of endothelial cells (Thornhill *et al*, 1992), which probably aids lymphocyte sticking and migration into tissues at sites of inflammation. This cytokine can also induce it's own synthesis at sites distant from infection/inflammation, possibly due to IFNγ present in the circulation or the presence of migratory cells which also produce this cytokine (Halloran *et al*, 1992).

Two other cytokines which have important immunoregulatory effects are IL-10 and IL-12.

Interleukin 10.

IL-10 is a cytokine which stimulates Th_2 like responses and is produced by activated $CD4^+$ $Th_{1,2,0}$, $CD8^+$ Th_2 like T cells, B cells and also activated monocytes and Mθs (Le Gros and Erard, 1994). The IL-10 molecule itself is (in human, mouse and rat) 160 aa long, with at least 73% identity between the three species at the aa level (Feng *et al*, 1993). The majority of the variation between these molecules arises from differences in glycosylation. The IL-10 receptor is approximately 110 kDa and expressed on monocytes/Mθs, B cells, thymocytes, endothelial cells, neutrophils, granulocytes and mast cells. The most potent immunoregulatory aspect of this cytokine would appear to be the effect which IL-10 exerts on APCs and in particular Mθs. Work has shown that IL-10 down regulates the expression of MHC class II, adhesion molecules such as B7/ICAM-1 and suppresses the expression of a number of proinflammatory cytokines including: IL-1; IL-6 and TNFα (Willems *et al*, 1994; Fiorentino *et al*, 1991a; de Waal *et al*, 1991). This cytokine also indirectly effects the production of cytokines from Th_1 like cells (Fiorentino *et al*, 1991b) through the down

regulatory effects which IL-10 exerts upon the APCs. It would therefore appear that IL-10 plays a major role in the regulation of immune functions and especially inflammatory reactions. Indeed it has been shown that mice made deficient in IL-10 by gene targeting show normal lymphocyte development but suffer inflammation of the bowel due to uncontrolled immune responses resulting from the high enteric Ag load (Kuhn *et al*, 1993).

Interleukin 12.

IL-12 also known as NK stimulatory factor is a cytokine comprised of two unrelated subunits. The smaller subunit (p35) shows homology to IL-6 and the larger subunit (p40) homology to IL-6R. This has lead investigators to speculate if IL-12 has evolved from an IL-6 cytokine/receptor complex (Stern *et al*, 1990; Wolf *et al*, 1991). The main cells producing this cytokine are monocytes/MΘs and B cells, with the 110 kDa receptor for IL-12 being expressed upon: NK cells; $CD4^+$ T cells; $CD8^+$ T cells. IL-12 stimulates both the growth and development of both $CD4^+$ and $CD8^+$ T cells and the production of predominantly a Th_1 like cytokine profile (Hsieh *et al*, 1993). The effect on NK cells is marked with increases being seen in proliferation, differentiation and activity. The presence and action of IL-12 has also been suggested to aid in monocyte/MΘ activation. The method relies on the induction of expression of IFNγ by IL-12 stimulated T or NK cells and so activates these myeloid cell indirectly (as shown in Fig 1.3).

MΘs and intracellular protozoan pathogens.

T.annulata sporozoites infect monocytes and MΘs, as do a number of protozoan parasites including: *Toxoplasma.gondii*; *Leishmania. spp*; *Trypanosoma cruzi*. In this section I shall highlight some of the recent relevant work in this area and compare it with the current knowledge of *T.annulata.*

Campbell *et al* (1994) showed that once infected with *T.annulata* the cells acquire an activated phenotype. However, when monocytes and MΘs infected with other intracellular protozoan parasites are activated it results in the destruction of the

parasite within the cell. This can be seen in Leishmanial infections (Howard, 1986; Ghalib *et al*, 1995). Work has shown that *Leishmania major* infections in certain strains of susceptible mice augment a Th_2 like reaction and that the immune responses induced are not capable of eliminating the protozoal infection (Cakkalath and Titus, 1994, Reiner *et al*, 1994). The study by Cakkalath and Titus (1994) also showed that the infected MΘs produced IL-1 but not IL-6 and that the IL-1 played a role in the augmentation of the Th_2 response. The fact that IL-6 was not produced suggests that the MΘs were not fully activated following infection with this parasite and that the presence of the parasite appears to force the production of a nonresponsive Th_2 reaction. Substantial work has been carried out upon *L. donovani* infections and again it has been shown that the leishmanial parasite within the MΘs can be removed by the induction of a Th_1 response, aided by treatment of cultures with IL-12 (Ghalib *et al*, 1995).

Toxoplasma gondii also infects MΘs and activation of cells infected with this protozoan also results in the destruction of the parasite (Remington and Krahenbuhl, 1982, Sibley *et al*, 1991). This is also the case for *Trypanosoma cruzi* grown in mouse peritoneal MΘs (Nogueira and Cohn, 1978). Thus protozoan parasites of MΘs are often susceptible to activation of their host cells and this is a major mechanism for the removal of intracellular organisms. However, at least one of these parasites (Leishmania) is able to alter the T helper cell response to evade killing. One interesting aspect of *T.annulata* infection is that infected cells not only appear to possess an activated phenotype but that they also induce the very type of T helper cell response that one would think would aid in the removal of the parasite (*eg*, a Th_1 type response). This is not seen to happen and suggests that this parasite also possesses the capability to manipulate the immune response to evade destruction.

This thesis details investigations into the MHC class II expression, T cell stimulatory ability and cytokine expression of *T.annulata* macroschizont infected cells and attempts to relate this to some of the properties of cell line vaccines.

Chapter II.

General Materials and Methods

Section 2.1

Production of T.annulata infected cell lines.

In vitro infection of bovine peripheral blood mononuclear cells with *Theileria annulata* sporozoites of the Ankara (Turkey) strain (Schein, 1975) and production of continuously growing infected cell lines and clones is outlined below.

Peripheral Blood Mononuclear Cells (PBM).

The PBM used during this study were isolated from an 8 month old Friesian calf (No.12929), as described by Glass and Spooner (1989). Whole venous blood was collected, using 20% ACD as an anticoagulant, layered over Ficoll-Hypaque S.G. 1.077 ("Lymphoprep", Nycomed) and centrifuged at 1500g for 25 minutes. The mononuclear cell layer was collected and washed in phosphate buffered saline (PBS) by centrifugation at 300g for 10 minutes. Cells were then washed twice more in PBS and finally in RPMI 1640 medium (Gibco BRL), centrifuging at 100g for 10 minutes. Cells were resuspended in TC medium (500ml RPMI 1640 (Gibco BRL) supplemented with 25mM Hepes, 10% FCS (Imperial Laboratories), 2mM L-Glutamine (Gibco BRL), 100 IU ml^{-1} Penicillin 100μg ml^{-1} Streptomycin (Gibco BRL)) counted and adjusted to the required concentration.

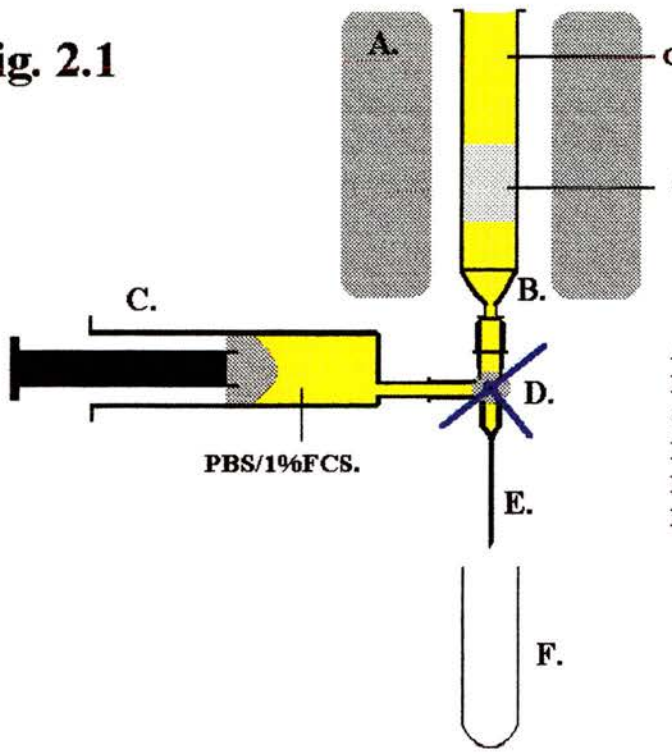
Magnetic Antibody Cell Sorting of $CD14^+$ monocytes-Principles of MACS separation.

$CD14^+$ cells were separated from PBM by the Magnetic Antibody Cell Sorting (MACS) System (Miltenyi, 1990). The principle of the MACS system is as follows: specific populations of cells are separated from PBM by positive selection following labelling with mAbs binding specific epitopes (*eg*, CD14). These primary mAbs are subsequently labelled with a secondary Ab conjugated to a magnetic tag (MACS beads).

Fig. 2.1 **Diagrammatical representation of Magnetic Antibody Cell Sorting (MACS) apparatus.**

Diagram of MACS cell separation apparatus, including - magnet (A), MACS column (B), syringe (C), three way tap (D)/needle (E) and collection tube (F). Cells tagged with primary mAbs and secondary mAbs/magnetic MACS beads are separated from unlabelled cell populations as the former adhere to the magnetised cell binding matrix of the MACS column. Unlabelled cells are then flushed away, labelled cells are subsequently harvested from the column after its removal from the magnet.

Fig. 2.1



A = MACS magnet.
B = MACS column.
C = 10ml syringe.
D = Three way stop tap.
E = Needle (variable gauge).
F = Collection tube.

The column (Fig 2.1) is placed between the poles of a powerful magnet and the plastic coated steel wool is conditioned with ice cold PBS/ 1% FCS before the addition of the cell suspension. This ensures that cell membranes are not damaged when in contact with the steel wool's surface.

The cell suspension is then run through the column. The speed of passage of the cells is controlled by a needle attached (via a three way stop tap) to the base of the column. The flow rate is altered by the use of needles with varying gauges. Magnetically tagged cells bind to the magnetised steel wool as they pass through the column and unlabelled cells are washed out of the column. The population of cells attached to the steel wool are then washed by removing the column from the poles of the magnet and backflushing the column with 10mls of chilled PBS 1% FCS from the syringe attached to the three way stop tap. The needle is then changed to a larger gauge to increase the flow rate through the column. The column is then placed back between the poles of the magnet and contaminating cells washed from the column, while the labelled cells adhere, once again to the steel wool. Once washed the cells can then be eluted from the column. The column is removed from the magnet and 10mls of chilled PBS/1% FCS used to flush labelled cells from the column. During the separation of $CD14^+$ cells used during this study, the cells were run through the MACS column, washed, eluted and passed back through the column to obtain maximum purity. The three cell populations (initial $CD14^-$ population, second $CD14^-$ "wash" population and the $CD14^+$ population following the second separation) obtained were analysed using flow cytometry to assess the purity of each population (data shown in Chapter III, Figs 3.1 a-d).

Cell labelling.

PBM were washed in chilled PBS/1% FCS. $2x10^8$ PBM were adjusted to $1x10^7$cells/ml labelled with a 1/200 (final) dilution of the mouse anti ovine CD14 mAb VPM65 (Gupta *et al*, 1996) for 20 mins on ice. Cells were then washed twice in chilled PBS/1% FCS and magnetically labelled using a rat anti mouse $IgG1_1$ MACS bead conjugate (sodium azide removed as per manufacturers instructions), for 20 mins

at 4°C (labelling is inhibited at temperatures below 4°C). The cells were diluted to 10^7 cells per 80µl PBS/1% FCS and 20µl of MACS beads added per 80µl. After a 20 minute incubation (4°C) cells were washed (100g, 10 mins) twice and resuspended in chilled PBS/1% FCS at $4x10^7$ cells ml^{-1}.

Column preparation.

During cell preparation and staining the MACS column was prepared. A B2 MACS column (Miltenyi Biotec), capable of retaining $1x10^8$ positive cells was prepared. The column was sterilised by filling with 70% ethanol from the tap/syringe assembly (Fig. 2.1) and allowed to stand for 10 mins. Following this the column was washed three times with 15mls of chilled PBS/1% FCS, then refilled and allowed to stand until used. The column was filled with freshly chilled PBS/1% FCS just before use, to cool the column prior to addition of the cells. Cells were then separated as detailed above using a 24 gauge needle for the first stage and a 22 gauge needle for the second.

T.annulata sporozoites.

T.annulata sporozoites were provided by the Centre for Tropical Veterinary Medicine, University of Edinburgh. Parasite was produced as ground up tick supernatant (GUTS) (Brown *et al*, 1983) and supplied as cryopreserved GUTS at 1 Tick equivalent (TE) ml^{-1} in 7.5% glycerol (BDH) and MEM/3.5% BPA.

In vitro infection of $CD14^+$ monocytes with sporozoites.

T.annulata infected cell lines were produced by infecting $CD14^+$ monocytes using cryopreserved GUTS as described by Brown *et al* (1983, 1989). Before infection, GUTS (1ml at 1TE/ml) were allowed to thaw rapidly and then equilibrate at room temperature for 20 minutes. The parasite material was then serially diluted in doubling quantities of TC medium containing 20% FCS to an approximate volume of 8ml, allowing 20 minutes between dilutions. The sporozoite containing medium was then added to $1\text{-}2x10^7$ $CD14^+$ monocytes in 2ml of TC. The flask was placed upright in a

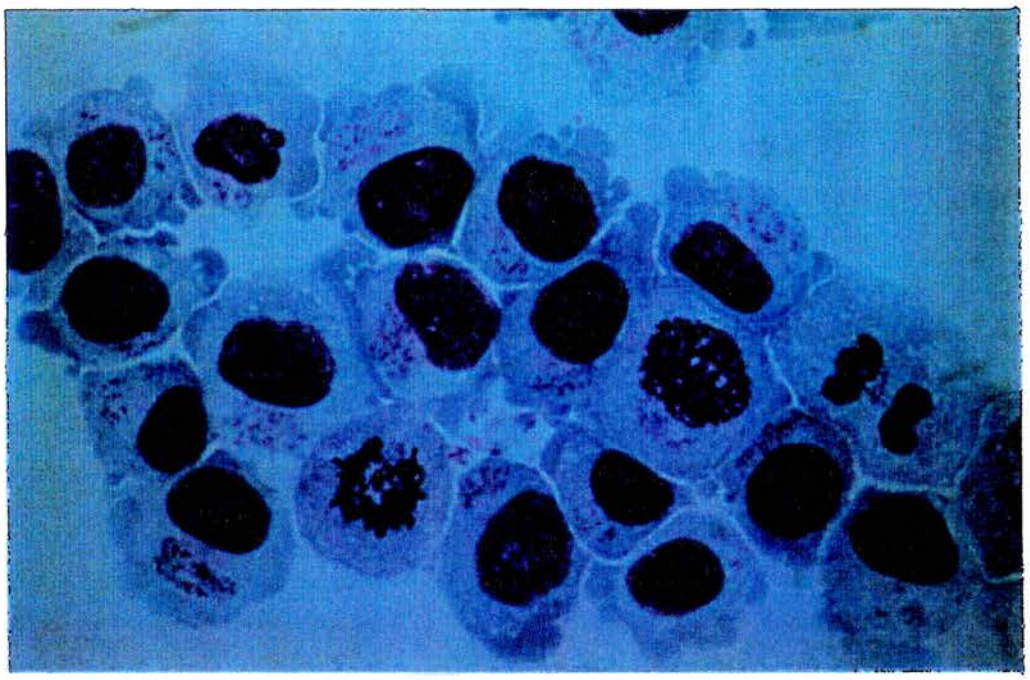
37°C, 5% CO_2 incubator for 24 hrs, 7ml of medium was carefully removed and replaced with 7ml fresh TC, and the flask placed horizontally.

Once an infected cell line had been established, fresh medium was added depending on the rate of growth of the culture (typically every 48-72hrs). Infection could be visualised using cytocentrifuge preparations (Cytospin II, Shandon). Here $1\text{-}2x10^5$ cells were centrifuged at 350rpm for 6 minutes using slide/filter assemblies (pre-wet with RPMI 1640 medium). The slides were then air dried, fixed in methanol, and stained for 30-40 minutes in Giemsa stain (Sigma) diluted 1:10 with "Gurr" buffer pH 7.2 (BDH). In well established cultures, >95% of viable cells could be identified as containing schizonts, as shown in Fig. 2.2.

Fig. 2.2 **A photograph of leucocytes infected with *T.annulata* macroschizonts.**

A typical cell line infected with *T.annulata* macroschizonts, visible as clusters of small pinkish dots within the cytoplasm but separate from the nuclei of the infected cells.

Fig. 2.2



Section 2.2

Cloning of T.annulata infected cell lines.

Clonal cell lines were produced by soft agar cloning (Cotton *et al*, 1973) of infected cells. 15ml Petri dishes (Nunc, Gibco, Paisley, UK) were covered with 10ml of TC medium containing 1% tissue culture grade agarose at 37^{0}C. Once this had cooled and solidified, 2ml *T.annulata* infected cells in TC medium (100 cells ml^{-1}) were mixed with an equal volume of culture medium/1% agarose suspension at 37^{0}C and layered over the solid agar. The suspension was left at room temperature for 30 mins to become semi-solid before incubating at 37^{0}C in 5% CO_2.

Petri dishes were examined daily, with discrete colonies first appearing at day 4. These colonies were transferred to separate wells of a 96 well round bottomed plate (Nunc, Gibco, Paisley, UK) containing 150µl of culture medium. Cells were incubated until the cell density approached 300-400 cells/well and transferred to a 24 well plate (Nunc, Gibco, Paisley, UK) and incubated until the cell density was approximately 2x10^{5}cells/well. Cells were then transferred to 10ml Nunc tissue culture flasks and treated in an identical manner to standard *T.annulata* cultures (Brown *et al,* 1983).

Mitomycin C treatment of PBM and T.annulata infected cells.

T.annulata infected cells were treated with Mitomycin C prior to use as stimulator cells. This compound prevents the cell growth and thus infected cells from overgrowing PBM cultures (Fraser *et al*, 1996). Mitomycin C ((Sigma) produced by the fungus, *Streptomyces caespitosus*)) cross links DNA and inhibits it's replication. PBM or *T.annulata* infected cells used to stimulate T cells were incubated in culture medium at 1x10^{7}cells ml^{-1} with 25µg ml^{-1} and 50µg ml^{-1} of Mitomycin C respectively (37^{0}C, 30 mins). Cells were then washed twice in RPMI 1640 (100g, 10 mins).

Section 2.3.

Proliferation assays.

T cell proliferation induced by the infected cell lines was measured as previously described (Glass & Spooner 1990). The *T.annulata* cells used in these experiments were passaged 24 hours prior to use, 2 mls of the cell culture was added to 8 mls of fresh medium. Briefly, autologous PBM ($4x10^5$/well) were incubated with mitomycin C treated autologous *T.annulata* infected cells ($4x10^4$/well) or 5μg/ml Con A for 5 days in 96 well flat bottom plates (Nunc, Gibco, Paisley, UK). Following a 6 hour pulse of tritiated thymidine (1μCi $well^{-1}$ [^{3}H]dThd, supplied by Amersham Int.) plates were harvested and counted as previously outlined using a Wallac MicroBeta and Cell Harvester).

Section 2.4.

Quantitation of MHC class II expression.

$F(ab')_2$ fragments of the anti ruminant, pan MHC class II mAb SW73.2, which is specific for nonpolymorphic epitopes on the β-chain of MHC class II molecules (Hopkins *et al*, 1986; Knowles *et al*, 1994), were iodinated with ^{125}Iodine (Amersham Int, Amersham, U.K.). Iodination was carried out as per the Pierce IODO-beads iodination protocol (Lee *et al*, 1984). During the process of radioiodination, radioactive ^{125}Iodine is introduced into tyrosine residues at positions ortho to the hydroxyl group of the residue. The mechanism of iodination used during IODO-bead protocol involves the oxidising agent *N*-chloro-benzenesulphonamide (sodium salt) (Markwell, 1982) covalently linked to nonporous polystyrene beads. This two phase iodination system allows removal of the bead from the protein suspension, thus limiting damage caused by extended contact with an oxidising agent.

$F(ab')_2$ Iodination.

One Iodobead was placed in a sterile eppendorf tube and washed with 500μl of PBS

(pH 6.5). The PBS was removed and 250µl of fresh PBS added, 0.6 mCi (approximately 6µl) of sodium ^{125}iodide was then added to the bead. The bead was incubated at room temperature for 5mins and 30µl of SW73.2 $F(ab')_2$ (14.5µg/10µl PBS) added. 164µl of PBS was added making a final volume of 450µl, followed by a 5 min incubation at room temperature. The $F(ab')_2$ suspension was removed from the bead to a sterile eppendorf and the bead washed in 50µl of fresh PBS, this was then added to the rest of the antibody suspension.

The $F(ab')_2$ suspension was passed down a PD-10 sephadex column (Pharmacia) pretreated with RPMI 1640/1% FCS (0.1% NaN_3). Fractions were collected in 0.5ml volumes and the ^{125}I content (cpm sec^{-1}) of the fractions gauged using a Mini Assay Type 6-20 γ counter (Mini Instruments). The ^{125}I labelled fragments were generally found to be in the 3rd- 5th fractions and the total volume varied from between 1500-1800µl. 1µl of the fragment suspension was removed and added to 1ml of ice cold 20% trichloroethanoic acid (TCA), following a 5 min incubation on ice. The $F(ab')_2$/TCA was centrifuged at 14000g for 5 mins and the supernatant removed. The amount of ^{125}I attached to 1µl of $F(ab')_2$ was assessed using a Wallac 1261 Multigamma. The specific activity of the labelled fragments were calculated as shown below.

Calculation of the concentration of $F(ab')_2$ fragments (as supplied).

(1) $F(ab')_2$ supplied at 1.45g/l and has a molecular weight of 70,000Da.

(2) Therefore $1.45/70000 = 2.07 \times 10^{-5}$ moles of $F(ab')_2$ molecules.

(3) $2.07 \times 10^{-5} \times 6.02295 \times 10^{23} = 1.25 \times 10^{19}$ $F(ab')_2$ molecules l^{-1} (1.25×10^{13} μl^{-1}).

(4) Therefore $1.25 \times 10^{13}/1.45 = 8.6 \times 10^{12}$ $F(ab')_2$ molecules μg^{-1}.

(5) Therefore 30µl (or 43.5µg) of $F(ab')_2 = 3.74 \times 10^{14}$ molecules (total $F(ab')_2$ iodinated).

(N.B. - During radiolabelling experiments the volume of eluent which the ^{125}I-SW73.2 was diluted in and therefore the concentration of iodinated $F(ab')_2$ fragments per labelling reaction was taken into consideration. Also two assumptions are made here,

Calculation of the specific activity of radiolabelled SW73.2 $F(ab')_2$ molecules.

(1) (Mean cpm of 1µl $F(ab')_2$/3) x 4 = dpm of 1µl of $F(ab')_2$ suspension.

(2) dpm of 1µl $F(ab')_2$/60 x volume of $F(ab')_2$ suspension/10^6 = Total MBq of ^{125}I-labelled $F(ab')_2$.

(3) Specific activity = total MBq/43.5 (µg $F(ab')_2$ iodinated)/37000 = µCi/µg $F(ab')_2$.

(N.B, cpm = counts min^{-1}, dpm = decays min^{-1}).

The ^{125}I labelled $F(ab')_2$ suspension was stored at 4^0C for up to 5 days.

MHC class II Quantitation.

T.annulata infected cell cultures were passaged the day before use, to maintain the cells in exponential growth phase. The expression levels of MHC class II molecules were measured by saturation binding using ^{125}I-$F(ab')_2$ fragments as previously described (Trucco *et al* 1981, Hopkins *et al* 1986). SW73.2 $F(ab')_2$ molecules have only one binding site for MHC class II molecules. One binding arm of SW73.2 binds a nonpolymorphic epitope on the beta chain of class II molecules, whilst the other binds an epitope expressed on the Y3 myeloma line used to immortalise the Ab producing B cell.

To ascertain the concentration of ^{125}I-SW73.2 which would bind all the MHC class II molecules present on infected cells, triplicate samples of 10^6 cells were incubated with 100µl samples of ^{125}I-SW73.2 at dilutions of 25, 50, 75 and 100% (100% being $F(ab')_2$/RPMI undiluted after collection from the PD-10 column). A 50% solution of ^{125}I-SW73.2 $F(ab')_2$ was found to bind all the MHC class II expressed by the uncloned infected cell line (Fig. 2.3). This cell line was used to assess saturation binding conditions as it was found (during preliminary flow cytometric analysis) to express the highest levels of any of the cell lines used in this study (Chapter III).

Fig. 2.3 shows the percentage of ^{125}I-SW73.2 suspension added to infected cells and also the number of MHC class II specific $F(ab')_2$ fragments contained in 100µls of $F(ab')_2$ suspension at each dilution. This takes into account the number of MHC class II specific fragments added to the column ($3.74x10^{14}$) and the volume of ^{125}I-SW73.2

also the number of MHC class II specific $F(ab')_2$ fragments contained in 100µls of $F(ab')_2$ suspension at each dilution. This takes into account the number of MHC class II specific fragments added to the column ($3.74x10^{14}$) and the volume of ^{125}I-SW73.2 containing liquid collected off the PD-10 column, (in this instance 1500µls) and gives a concentration of $2.49x10^{13}$ class II specific molecules per 100µls of undiluted eluent. During binding assays triplicate samples of $1x10^6$ cells of different *T.annulata* infected cultures were labelled on ice for 30 mins with 50µl of ^{125}I-$F(ab')_2$/RMPI 1640 (0.1% NaN_3), plus 50µl of RPMI 1640. Unbound $F(ab')_2$ was separated from the cells by washing with 3 x 750µl aliquots of chilled PBS followed by centrifugation at 1000 rpm for 2 mins (Eppendorf centrifuge). Cells were pelleted after the third wash (centrifugation at 10000 rpm, 2 mins (Eppendorf centrifuge)) and the supernatant removed. The amount of ^{125}I-SW73.2 bound to the cells was assessed by direct γ counting using a Wallac 1261 Multigamma. The numbers of MHC class II molecules per cell were calculated as shown below.

(1) µCi of labelled cell sample/specific act = µg of class II specific $F(ab')_2$ molecules bound to labelled cells.

(2) µg $F(ab')_2$ bound x $8.60x10^{12}$ = No. $F(ab')_2$. ($8.60x10^{12}$ = No. $F(ab')_2$ μg^{-1}).

(3) No. $F(ab')_2/1x10^6$ = No. $F(ab')_2$ $cell^{-1}$ = No. MHC class II molecules $cell^{-1}$.

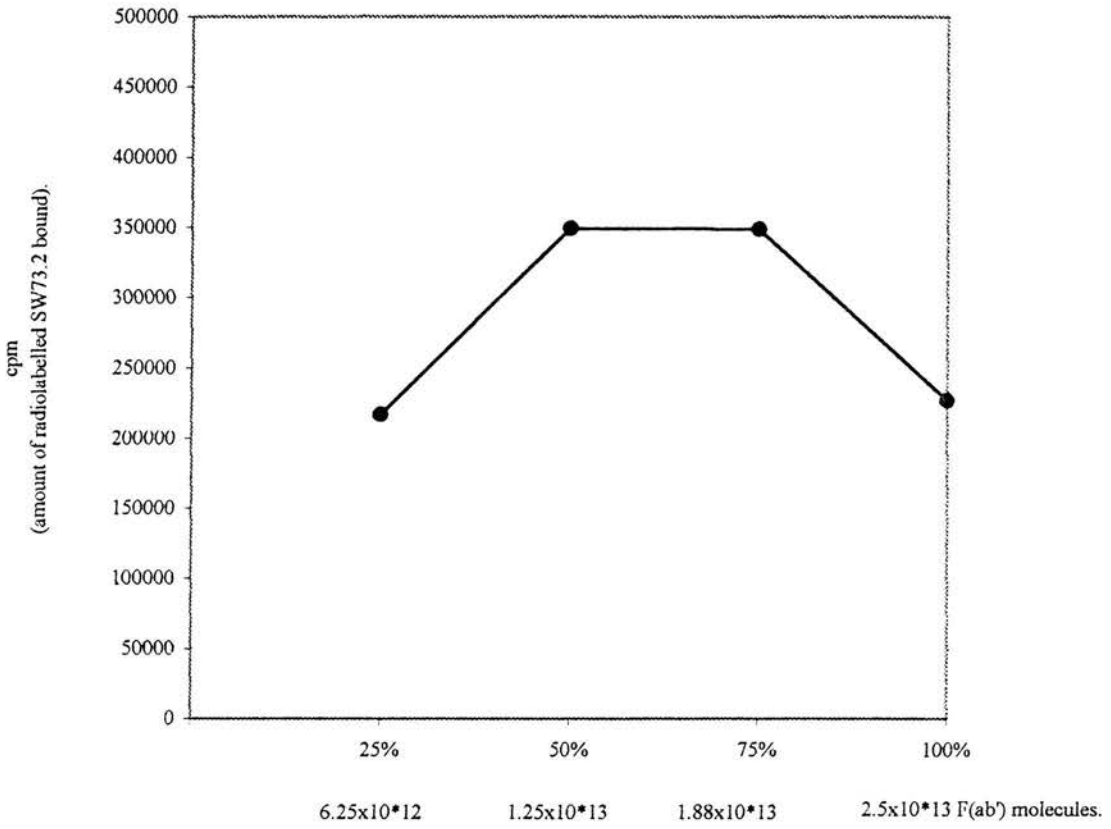
(N.B. $8.60x10^{12}$ = MHC class II specific $F(ab')_2$ molecules per µg, $1x10^6$ = total number of cells labelled.

Fig. 2.3 **Assessment of the concentration of ^{125}I labelled SW73.2 $F(ab')_2$ fragments necessary to bind the MHC class II expressed by 10^6 *T.annulata* infected cells under saturating conditions.**

Data from a saturation binding assay where ^{125}I-SW73.2 $F(ab')_2$ fragments were used to assess the amounts of radiolabelled anti-pan MHC class II antibody fragments necessary to bind MHC class II expressed by *T.annulata* infected cells under saturating conditions. Triplicate samples of 10^6 macroschizont infected cells were incubated for 30 min on ice with varying concentrations of ^{125}I radiolabelled SW73.2 $F(ab')_2$ fragments. Three washes with ice cold PBS removed unbound fragments and radiolabelling of cell samples was then assessed by γ-counting. A 50% solution containing $1.25x10^{13}$ $F(ab')_2$ fragments was found to bind all MHC class II molecules expressed by 10^6 infected cells.

Fig. 2.3

Assessment of radiolabelled SW73.2 F(ab') fragment concentration needed to bind MHC class II molecules under saturation conditions.



Section 2.5.

Flow cytometric analysis.

Flow cytometric (FC) analysis of *T.annulata* infected cells was performed to assess phenotype and the expression of MHC class I and class II molecules (Campbell, 1995). Analysis was performed using the mAbs shown in Table 2.1 and the secondary conjugates shown in Table 2.2. Primary mAbs and secondary conjugates used in FC analysis were titrated before use to determine optimal concentrations, primary mAbs were used at 1:1000 (final concentration of diluted ascites) and secondary conjugates were used at concentrations of 1:100 (final concentration).

Table 2.1

mAb (isotype).	Specificity.	Reference.
VPM36 (IgG_1).	MHC class II (DQ).	Dutia *et al*, 1993; Dutia *et al*, 1995.
VPM54 (IgG_1).	MHC class II (DR).	Dutia *et al*, 1993; Dutia *et al*, 1995.
VPM65 (IgG_1).	CD14.	Gupta *et al*, 1996.
IL-A21 (IgG_{2a}).	MHC class II.	Davis *et al*, 1994.
IL-A24 (IgG_1).	Macrophages.	Ellis *et al*, 1988.
IL-A109 (IgM).	Monocytes.	MacHugh *et al*, 1990.

Table 2.1 **Primary monoclonal antibodies used during flow cytometric analysis.**

Details of the mAbs used during this study including, isotype, specificity and reference.

Table 2.2.

Immunoconjugate	Working Dilution
Rabbit anti-mouse (MAR) Ig-FITC	1:100

Table 2.2 Fluorochrome conjugated secondary antibody.

Details of the secondary antibody (supplied by Sigma) used during this study. The secondary used was a fluorescein isothiocyanate (FITC) conjugated Ab.

Staining of cells for flow cytometric analysis.

Cells were prepared for FC analysis by washing twice in Cell Wash (PBS/NaN_3 (pH 7.2) Becton Dickinson) and resuspended in chilled cell wash (supplemented with 5% γ-globulin free horse serum) at $1x10^7$cells ml^{-1} (FC assays were performed at 4^0C). Aliquots of 50μl of cell suspension ($5x10^5$ cells) were added to separate wells of a 96 well round bottom plate and 50μl of each mAb added to a well. Negative controls consisted of cells incubated with normal mouse (1:1500, Sigma), followed by secondary conjugate. Cells were incubated on ice for 30 mins, unbound mAb molecules were removed from cells by washing 3x with 150μl of chilled cell wash (plates were centrifuged at 100g for $2^1/_2$ mins at 4^0C). Cells were then resuspended in 50μl of the appropriate secondary mAb conjugate and incubated (covered from light) on ice for a further 30 mins. The cell samples were then washed a further 3x as before, resuspended in 100μl of cell wash and covered from light until use.

FACScan settings and operation.

A FACScan (Becton Dickinson) was used for all FC analysis. Data acquisition and analysis was performed using Lysis II software (Becton Dickinson). The FACScan flow cytometer uses a single argon laser to produce a beam of light (λ = 488nm),

which is used to assess the fluorescence, size and internal complexity of cells passed through the FACScan. As cells pass through the path of the light beam, changes in the beam's pattern/intensity are recorded by photomultiplier tubes and converted into electronic impulses. These impulses are then processed by the FACScan and are used to produce the data shown on the monitor. The parameters assayed by this method are the fluorescence of conjugate labelled cells and their size/complexity.

Calibration of the FACScan prior to data acquisition is achieved using the negative control samples (cells stained with secondary Ab and mouse serum only). The effect that the cells have on the path of the laser beam is used to assess the size and complexity of the cells. The presence of the cell within the light beam causes the beam to bend, the larger the cell, the higher the degree of bending. The degree of bending of the light relates to the amplitude of the light scatter. Changes in this parameter are recorded as forward scatter (FSC) along the axis of laser emission and give an indication of the size of the cells passing through the beam. The degree of scattering of the light beam relates to the complexity or degree of organisation within the cells. Side scatter (SSC) is caused by the cells' ultrastructure and organelles refracting the light beam as they pass through. The light is refracted at 90^0 to the angle of incidence, detectors record these emissions and convert them to electronic impulses. High SSC values indicate high levels of internal organisation within cells, *eg T.annulata* infected cells show higher FSC and SSC readings than uninfected PBM. Data produced is plotted as a dotplot and live cells are gated on a linear scale to remove dead cells and red blood cells.

Fluorochrome conjugates passing through the laser beam absorb light at 488nm and emit light at an altered wavelength. Fluorescein isothiocyanate (FITC) emits light at 530nm and Phycoerythrin (PE) emits light at 580nm. Florescence is measured on $\log_{10}$ scales, with settings typically between 580-620meV (FITC, FL1) and 500-540meV (PE, FL2). The transmitted light of these differing wavelengths are measured by detectors on the FACScans optical filters, the amount of emitted light detected is proportional to the amount of fluorochrome bound to the cell surface.

Data acquisition was carried out in Lysis II acquisition mode, with 10000 cells taken

for each sample. Control fluorescence levels were obtained from the negative control samples. Data was analysed using either Lysis II at the FACScan work station or PC Lysis (Becton Dickinson) operating in Microsoft Windows and was presented as either histograms of cell frequency against $\log_{10}$ fluorescence or dotplots of $\log_{10}$ fluorescence against FSC or SSC.

Molecular biological techniques used to assess cytokine expression.

Section 2.6.

RT-PCR analysis of cytokine transcripts from T.annulata infected cell lines.

RNA Extraction.

Total cell RNA was isolated from various *T.annulata* cell lines, clones and T cells using RNeasy method (Quiagen) according to the manufacturers instructions. This method does not require the use of chloroform/guanidinium isothiocyanate extraction and can be carried out at room temperature in approximately 20 mins. The method relies on the fact that RNAse enzymes are unable to function at the high salt concentrations in the lysis buffer.

Homogenisation of the cells in a solution of guanidinium hydrochloride, by repeated passage through a syringe and fine needle, bursts the cells and shears nuclear DNA. Addition of 70% ethanol precipitates the RNA, which binds to the RNeasy column's silica based membrane. Contaminating material is removed during subsequent washes. The purified RNA is then eluted by addition of double distilled/deionised/autoclaved water (DDA H_2O) and brief centrifugation.

RNA preparation Protocol.

10^7 cells were pelleted at 100g for 10 mins and the supernatant removed. Cells were resuspended in 350µl of lysis buffer (plus 10µl tissue culture grade 2-mercaptoethanol ml^{-1} lysis buffer (Sigma)) and homogenised by passing the suspension rapidly back and forth 20-25 times through a 21 gauge needle and 1ml syringe. 350µl of 70%

ethanol was added to the lysate and mixed using a 1ml pipette (Gilson). The 700µl of lysate was added to an RNeasy column and centrifuged at 10000 rpm for 15 seconds in an Eppendorf centrifuge. The supernatant was then removed from the collection tube and 700µl of wash buffer No.1 added, the tube was then centrifuged at 10000 rpm for 15 seconds. The collection tube was then discarded and a fresh 2 ml collection tube placed on the column, 500µl of wash buffer No.2 was then added and the column centrifuged as before with the supernatant being discarded. These buffers are designed to wash unwanted protein off the membrane, away from the RNA.

A further 500µl of wash buffer No.2 was added to the column followed by centrifugation at 14000rpm for 2 mins. The column was then transferred to a fresh 1.5 ml eppendorf tube and 40µl of DDA H_2O added to the column, which was incubated on ice for 5 mins. The column was then centrifuged at 10000 rpm for 1 min (Eppendorf centrifuge) and the resulting 40µl of RNA suspension placed on ice.

The total RNA yield was assessed by U.V spectrophotometry at 260nm and 280nm (Sambrook *et al*, 1989). 1µl of the 40µl RNA suspensions was diluted 1:100 in DDA H_2O, U.V absorbtion (Abs) was determined using a Beckman DU-62 spectrophotometer with a 100µl quartz cuvette. The apparatus was calibrated against DDA H_2O and then the Abs at 260nm and 280nm (Abs_{260}, Abs_{280}) were taken for the diluted RNA samples. The ratio of Abs_{260} to Abs_{280} gives an indication of the condition of the RNA sample. A ratio of approximately 2_{260}:1_{280} suggests an RNA sample with little nucleic acid degradation or contaminating protein. An Abs_{260} value of 1 corresponds to an RNA concentration of 40µg ml^{-1}, the concentration (µg ml^{-1}) of RNA was calculated by multiplying the Abs_{260} value by 40 (and the suitable dilution factors).

Reverse transcription of RNA to cDNA.

Samples of 5μg RNA were used for reverse transcription to cDNA using the SuperScript preamplification system (Life Technologies Ltd.) with oligo dT as a primer, according to the manufacturer's instructions.

Samples of 5μg of total RNA were placed in 500μl PCR tubes and a volume of DDA H_2O added to make a final volume of 12μl. 1μl of oligo dT primer (500μg ml^{-1}) was added to the RNA suspension. This mixture was incubated at 70°C for 10 mins on an Omnigene PCR machine (Hybaid) and then chilled on ice. This allowed the RNA to separate and the oligo dT to bind to the poly A tails of the mRNA molecules, with the exclusion of the other RNA molecules present. The tubes were then centrifuged (10000 rpm, 15 sec (Eppendorf centrifuge)) and the following superscript mix added to each sample.

Superscript mix:-	4μl	5x synthesis buffer.
	2μl	0.1M Dithiothreitol (DTT/reducing agent).
	1μl	10mM dNTP mix.
	1μl	200u μl^{-1} superscript reverse transcriptase.

Samples were then centrifuged at 10000 rpm for 15 sec and incubated at room temperature for 10 mins. The samples were then heated on an Omnigene PCR machine (Hybaid) at 42°C for 50 mins (allowing synthesis of the cDNA, followed by 90°C for 5 mins. This final step allowed the denaturation of the superscript enzyme. The samples were then placed on ice for 5 mins and centrifuged at 10000 rpm for 15 secs. cDNA samples were either used immediately after synthesis or stored at -20°C.

Primer design and manufacture.

Oligonucleotide primers were designed using the EMBL data base and the FASTA and PCRPLAN PC/GENE (version 6.7, Intelligenetics Inc. California. U.S.A.) computer programmes. The sequences of the mRNA transcripts of the bovine cytokines were located on the EMBLE data base (Switzerland). The majority of the

primers shown below were produced specifically for this study. However, the primers used to detect β-actin, IL-2, IL-2R, IL-4 and IFNγ were designed by G. Russell and J. D. M. Campbell (Roslin Institute) (Van Lerope *et al*, 1995).

Table 2.3

Cytokine sequence.	**EMBL database accession numbers.**
β-actin.	K00622.
IL-1α	X12497.
IL-1β	X12498.
IL-2	M12791, M13204, M17428.
IL-2R	M20818.
IL-4	M77120.
IL-6	X57317, S49716.
IL-10	Z29326.
IL-12 (p40)	BT14416, U11815.
TNFα	Z14137.
IFNγ	M29867.

Table 2.3 Cytokine sequence references.

Details of the cytokine primers made for this study and the accession number of the cytokine sequences used to design these primer sequences.

Using the PC gene program these sequences were analysed to locate suitable areas of cytokine mRNA sequence which could be used as the basis for oligonucliotide primers. When more than one sequence was found for a particular bovine cytokine, the sequences were compared and primers were designed to sit in areas which matched for all known sequences. The parameters used to select these primer pairs were as follows: primer lengths of no more than 20 bases; annealing temperatures of between 55-60^{0}C; GC contents of approximately 50%; exclusion of self binding sites. Once primer pairs were selected their sequences were checked for homology to the known cytokine sequences and other known sequences using the FASTA computer programm. Oligonucliotide primers which also showed homology to other known sequences were discarded and only primers which primed from specific cytokine templates were selected for use. Primer pairs were manufactured by Oswel DNA Service (Dept of Chemistry, Edinburgh University). Oligos were supplied at approximately 0.1nM μl^{-1}, diluted to a working concentration of 25pM μl^{-1} and stored at -20^{0}C, stock solutions were aliquoted and stored at -80^{0}C.

Polymerase chain reaction analysis of cDNA.

Polymerase chain reaction (PCR) reactions were performed using 2μl of the resulting cDNA. Primers specific for bovine cytokine sequences (Tables 2.4a and b) were used to amplify cytokine cDNA, during a 30 cycle PCR program (Taq polymerase and buffers from Gibco BRL). The PCR mix used in each 30μl reaction contained; 19μl DDA H_2O, 3μl 10x PCR buffer (Gibco BRL), 1.2μl MgCl (50mM, Gibco BRL), 2.4μl dNTP mix (dATP, dCTP, dGTP, dTTP, each at 10mM, Pharmacia), 1μl +ve primer (25ρM), 1μl -ve primer (25ρM), 2μl cDNA suspension, 0.4μl (2 units) *Thermus aquaticus* Taq polymerase (Gibco BRL). Sample tubes (500μl PCR tubes, Robbins Scientific Corporation) were centrifuged at 10000 rpm for 15 sec and then a thin layer of liquid paraffin added to prevent evaporation during the PCR program.

The PCR programm was carried out on an Omnigene PCR machine (Hybaid), the following PCR program was used.

Stage 1	95^{0}C x 3 mins.	(denaturation step).
Stage 2	95^{0}C x 1 min.	(denaturation step).
	55^{0}C x 1 min.	(primer attachment step).
	72^{0}C x 1 min.	(elongation step).
	(Stage 2 cycles 30 times).	
Stage 3	72^{0}C x 5 mins.	(elongation step).

Hold temp = 20^{0}C.

Following the PCR reaction samples either underwent electrophoresis or were stored at -20^{0}C until required.

Table 2.4a.

mRNA.	Primer direction.	SIZE bp.	T_m at 0.1M Na^+ ions.	5'-SEQUENCE-3'
β-actin	+	288	61	CTGGCACCACACCTTT TACAACGAG
	-		61	AGCCAAGTCCAGACG CAGGATG
IL-1α	+	332	59	TCACCGATGATGACCT GGAAGCC
	-		59	GATTTTGGGTGTCTCA GGCATCTCC
IL-1β	+	432	63	CCGACGAGTTTCTGTG TGACGCACC
	-		61	CGAAAATGTCCCAGG AAGACGGGC
IL-2	+	255	51	AAGTCATTGCTGCTGG ATTTAC
	-		52	CCTGTAGTTCCAAAAC GATTCTC
IL-2R (CD25)	+	325	63	GCCAACAAGAGGCTG AAAGGAAACCC
	-		63	TGCCCCAGCGTGAAAT GGTAGAC

Table 2.4a Cytokine primer sequences.

Details of PCR primers specific for β-actin, IL-1α, IL-1β, IL-2 and IL-2R, including, direction, size of product in base pairs, melting temperatures and nucleic acid sequence.

Table 2.4b

IL-4	+	427	58	GCATTGTTAGCGTCT CCTGGTAAAC
	-		55	CTTCATAATCGTCTT TAGCCTTTCC
IL-6	+	655	54	ATGAACTCCCGCTTC ACAAGC
	-		50	TACTTCATCCGAATA GCTCTC
IL-10	+	733	48	GCTCAGCACTACTCT GTT
	-		49	GTTCACAGAGAAGC TCAGT
IL-12	+	574	60	CCTGTCACAAAGGA GGCGAGGC
	-		61	CGTCAGGGAGAAGT AGGAATGCGG
TNFα	+	500	54	CTCAGGTCATCTTCT CAAGCC
	-		61	CAGGGCGATGATCC CAAAGTAGACC
IFNγ	+	531	57	GGAGCTACCGATTTC AACTACTCCG
	-		56	GCAGGCAGGAGGAC CATTACG
G3PD	+	565	57	GATGCTGGTGCTGA GTATGTAGTG
	-		57	ATCCACAACAGACA CGTTGGGAG

Table 2.4b Cytokine primer sequences.

Details of PCR primers specific for IL-4, IL-6, IL-10, IL-12, TNFα, IFNγ and G3PD, including, direction, size of product in base pairs, melting temperatures and nucleic acid sequence.

The products of the RT-PCR reactions were visualised (under ultraviolet light) on a 2% agarose (Sigma, type 1-A)/1x TBE (Tris/Boric acid/EDTA) gel, containing 0.1μg/ml Ethidium Bromide. Before loading of the samples 15μl of sample was added to 5μl of loading buffer (80% glycerol, 0.02% bromophenol blue, 19.98% Tris:EDTA). Gels were electrophoresed at 100v for 1.5-2 hours in 1xTBE running buffer. Polaroid photographs were taken to record the resulting gels. β-actin or Glucose-3-phosphate dehydrogenase (G3PD) primers were used as positive controls during these reactions. G3PD primer sequences were supplied by I.L.R.I. (Kenya), these primers proved a more reliable control than the original β-actin control.

Restriction digest analysis of PCR products.

Restriction maps of the PCR products were calculated from the known bovine cytokine sequences when cut with specific restriction enzymes, chosen using the PC Gene computer program. 15μl of the PCR products were digested with selected restriction enzymes, the fragments were visualised as previously outlined. Production of fragments the size of which matched those predicted allowed the authenticity of the PCR reactions to be assessed. Five enzymes were used: Taq 1 (Pharmacia), Pvu II (Boehringer-Mannheim), Bgl II (Boehringer-Mannheim), Hae III (Biolabs), Pst 1 (Pharmacia).

Semi-Quantitation of mRNA.

Limiting cycle PCR was performed to ascertain the relative expression levels of the various cytokine mRNAs between different clones, cell lines and stimulated T cells. In all experiments 5μg of RNA was used to synthesise cDNA and 2μl of cDNA suspension was used as templates for the primer pairs in each PCR reaction. This means that the amount of mRNA present before reverse transcription correlates to the amount of PCR product synthesised during the PCR reactions. The presence of high levels of cytokine mRNA in a cell at the point of RNA extraction, means the PCR product produced from the resulting cDNA templates will be visible at a lower number of PCR cycles than for cells with lower levels of mRNA present at the point of RNA extraction.

The PCR protocol remained the same apart from the number of cycles used to amplify each 2µl of cytokine cDNA. Cycle numbers ranged from 20 to 30, in increments of 2 cycles. Comparison of the cycle at which the PCR products became visible under U.V light were used to assess the relative expression of the cytokine mRNA species. Gels were cast, electrophoresed and visualised as previously described.

In vivo assessment of clones I and L.

Section 2.7.

Immunisation trial of clones I and L.

Based on the *in vitro* data (contained in chapters III and IV) produced from studies of the parent line and the clones, clone I and L were selected for the immunisation of six three year old cows. The aim of this experiment was to compare the effects of these different clonal cell lines upon the subject animals. Three animals were immunised with clone I and three immunised with clone L and progression of infection and the health of the animals followed for 30 days post infection. Data gathered included; daily temperatures, packed cell volume (PCV), total erythrocyte count (TEC), total leukocyte count (TLC), the time of appearance of macroschizont infected cells and piroplasms were also noted. Attempts were also made to isolate and grow macroschizont infected cells from each animal and to compare the phenotypes of any lines produced to the original clone.

Immunisation with macroschizont infected cells.

Clones I and L were passaged the day prior to immunisation. Immediately before immunisation, rectal temperatures and 2 ml EDTA venous blood tubes (Vacutainer, Becton Dickinson) were taken from each animal and notes taken of, PCV, TEC and TLC. Each animal was immunised (subcutaneously in the shoulder) with $1x10^6$ macroschizont infected cells of either clone I or clone L diluted in TC culture medium. Post immunisation rectal temperatures and 2 ml EDTA venous blood tubes were taken daily. Cattle suffering fever (temperatures over 39.5^0C) or severe clinical symptoms (such as drastically reduced PCVs) for two or more days were treated with the quininoid drug Butalex (2.5mg kg^{-1}; Mallinckrodt Veterinary Ltd). Data collection from these animals was terminated 23 days post immunisation.

Analysis of 2 ml EDTA blood tubes.

The 2 ml EDTA venous blood samples were used to assess the effect of immunisation of the animals with the clones, upon the circulating erythrocyte and lymphocyte pools. PCV was calculated using a Hawksley Microheamatocrit centrifuge and Hawksley reader (Hawksley & son Ltd, Sussex). TLCs and TECs were calculated using a Coulter Counter (Coulter Electronics). Blood was diluted to aid accurate counting, with 20μl of blood being aspirated into a Dual Diluter III (Coulter Electronics) with the addition of 20mls of Isotone (Coulter Electronics). This gives a dilution factor of 1:1000, for lymphocyte counting. To assess RBC numbers, 100μl of diluted blood was aspirated into the Double Diluter and 20ml of isotone were added, resulting in a final dilution of 1:200000. Six drops of Zap-Globin (Coulter Electronics) were added to the TLC samples and the suspension shaken. Zap-globin lyses erythrocytes for accurate leucocyte counting.

The Coulter Counter (Coulter Electronics) used to obtain TLCs and TECs was zeroed with 10 mls of fresh Isotone and the TEC samples passed through the machine, 0.5ml of each sample was aspirated and TEC noted. Following this the TLC samples were analysed using the same procedure. The counter probe had a 100 μm orifice and probe blockages (due to dust of aggregates of cells) were cleared using fresh Isotone and a soft brush. Each sample was counted twice and the mean number of cells counted calculated. Taking into consideration the dilution factors of the samples and the volume of each sample aspirated, the number obtained for each sample corresponded to $Xx10^3$ cells ml^{-1} for TLCs and $Xx10^6$ cells ml^{-1} for TECs (X = number of cells counted).

Detection of macroschizont and piroplasm infected cells.

The appearance of macroschizont infected cells was assessed by microscopical examination of cytospin slides of PBM. Here $1\text{-}2x10^5$ cells were centrifuged at 350rpm for 6 minutes using slide/filter assemblies (pre-wet with RPMI 1640 medium) and a Cytospin II (Shandon). The slides were then air dried, fixed in methanol, and stained for 30-40 minutes in Giemsa stain (Sigma) diluted 1:10 with "Gurr" buffer pH

7.2 (BDH). Detection of piroplasms was carried out by microscopical analysis of air dried blood smears, fixed in methanol also stained in Giemsa.

Isolation of infected cells from animals immunised with clones I or L.

Infected cells were isolated from immunised animals using the same method employed to isolate PBM from whole blood. Sterile venous blood was layered over Lymphoprep (Nycomed), centrifuged and washed. The isolated PBM/infected cells were then placed in TC medium and cultured in 6 well plates (Nunc) at 37^0C/5% CO_2. Daily inspections were carried out to check for the presence of macroschizont infected cells. These cells appear as large blasting cells which begin to form clumps within the surrounding PBM. As infected cells overgrew the residual uninfected PBM, the cultures were split and the dividing cells supplied with fresh medium. Once cell density had reached approximately $5x10^6$-$1x10^7$ infected cells $well^{-1}$, the cultures were transferred to 10ml culture flasks (Nunc) and treated as normal *T.annulata* infected lines.

MHC class I /II typing and microsatellite analysis of infected cells.

The MHC class I & II types of vaccinating cell lines (clones I and L) and macroschizont infected cells reisolated from animals after immunisation were analysed as described in Spooner *et al* (1979) and Oliver *et al* (1989). Microsatillite analysis was conducted by Mrs Janis Barr (Ziegle *et al*, 1992).

Challenge of immunised animals with T.annulata sporozoites.

Thirty days after the end of the initial immunisation trial, the six experimental animals and two unimmunised control animals were each challenged with a potentially lethal dose of 1 tick equivalent (1TE) of cryopreserved *T.annulata* sporozoites (Gharb stock). Sporozoites were administered subcutaneously into the prescapular region. After inoculation clinical signs were monitored as previously detailed.

Statistical analysis of data obtained from immunisation study.

Statistical analysis was carried out with the aid of Mr D. Waddington, a statistician at The Roslin Institute. Data were analysed using one and two sided t-tests carried out on the Minitab computer program. The trial period of 23 days was divided up into 3 periods (days 1-10, 11-17, 18-23). This was based upon previous work which Mr Waddington had conducted with Dr A. Nichani into the progression of the disease. The three periods represent - (1) initial phase of infection (trends in this period were studied using regression analysis, followed by a double sided T test), (2) the phase during which signs are seen and (3) recovery phase. One sided t-tests were conducted when the outcome of a particular part of the experiment been predicted. Two sided t-tests were used to assess data when an outcome had not been predicted prior to data collection. Data from the following days (11-17/18-23) were analysed by calculating the mean values for the various parameters over these periods and performing single sided t-tests on the resulting values. For instance, the mean temp of group I (days 11-17) v.s. the mean temp of group L (days 11-17).

Chapter III.

Quantitation of MHC class II expression by *Theileria annulata* macroschizont infected cells and correlation with T cell activating ability.

Section 3.1

Introduction.

Once infected with *T.annulata* sporozoites, cells form continuously growing, macroschizont infected, MHC class II^+ cell lines (Spooner *et al*, 1989; Glass *et al*, 1989). The exact identity of the cells infectable with *T.annulata*, which can then form continuously growing cell lines has been a much investigated subject for some years. The identity of the cells infected *in vivo* remain unknown, during the last 6 to 8 years work concerning the identity of cells which become infected *in vitro* has provided strong suggestive evidence for the identity of the cells.

Work in this laboratory (Spooner, *et al*, 1989; Glass *et al*, 1989) showed that of mononuclear cells present in peripheral blood, those most easily infected and transformed *in vitro* were MHC class II positive monocytes/MΘs and B cells. Later work by Campbell *et al* (1994) narrowed down the range of phenotypes which were infectable *in vitro*, studying the infectability of elastin receptor positive and lipopolysaccharide (LPS) receptor positive ($CD14^+$) cells (of the monocyte/MΘ lineage). During this work Dr Campbell and myself performed studies into the infectability of these cells and also into the infectability of purified B and T cells. From the findings of these investigations we confirmed that the cells infected and transformed *in vitro* with the highest efficiency by *T.annulata* sporozoites were of the $CD14^+$ monocyte/MΘ lineage.

Log_3 dilutions of peripheral blood mononuclear cells (PBM), T cell, B cell, elastin $receptor^+$ and $CD14^+$ cells were prepared and incubated with *T.annulata* sporozoites (Brown *et al*, 1987). Elastin $receptor^+$ cells were included in this study after work by Williamson *et al* (1989) and Hall *et al* (1992) suggested that the elastin receptor was

a possible entry ligand for *T.annulata* sporozoites. Investigation of sporozoite surface antigens revealed a molecule (SPAG-1) expressed by the infective stage of the parasite contained an amino acid motif (Val-Gly-Val-Ala-Pro-Gly) characteristic of the elastin molecule. It was then postulated that the parasite used this elastin like molecule to bind to and infect elastin receptor$^+$ cells. However, data produced during *in vitro* infection studies showed that T and B cells did not become infected/"transformed" by sporozoites of *T.annulata* and the infectivity of elastin receptor$^+$ and elastin receptor$^-$ cells did not exhibit significant differences. The ratios at which CD14$^+$ cells infect and "transform" *in vitro* were 1:116 for CD14$^+$ cells and 1:6897 for CD14$^-$ cells (Campbell *et al*, 1994). CD14$^+$ cells, encompassing all monocytes and MΘ, are therefore the principle cells transformed by *T.annulata* sporozoites *in vitro*.

During the studies conducted into the infectibility of MHC class II positive cells and studies of the MHC class II expression of these cells after infection, it was observed that *T.annulata* infected cells generally expressed higher levels of class II molecules than uninfected cells (Glass and Spooner, 1990a). Infected cells act as functional Ag presenting cells, presenting Ag via MHC class II to CD4$^+$ T cells. However, Glass & Spooner (1990a) also demonstrated that infected cells possessed "augmented Ag presentation functions" as infected cells were capable of inducing activation of T cells from animals which had not been exposed to theileria.

Proliferation of PBM when cultured with infected cells (Rintelen *et al*, 1990; Glass and Spooner, 1990a) is associated with the activation of CD4$^+$ T cells (Campbell *et al*, 1995; Campbell, 1995) and is totally dependent on contact between infected cells and T cells (Campbell *et al*, 1995). *In vitro,* T cells activated by *T.annulata* infected cells do not form anti-theilerial responses (Nichani, 1994; Campbell, 1995). The "non-specific" T cell activation observed *in vitro*, also occurs *in vivo*, with T cells becoming activated following contact with infected cells within the draining lymph node (Campbell *et al*, 1995). These *in vivo* activated T cells are again not functional (Nichani, 1994; Campbell, 1995). Understanding the mechanisms by which *T.annulata* infected cells cause "non-specific" T cell activation are therefore of great importance in understanding mechanisms of pathogenesis.

MHC class II expression is essential for the Ag-specific activation of CD4$^+$ T cells

(Harding and Unanue, 1990). *T.annulata* infected macrophages express high levels of class II and possess augmented Ag presenting functions - the "non-specific" activation of autologous T cells. Thus Glass and Spooner (1990a) suggested that the T cell stimulatory ability of infected cells may be related to the high MHC class II expression of infected cells.

This chapter investigates the relationship between the MHC class II expression of *T.annulata* infected cells and their T cell stimulatory abilities. To further characterise the high levels of MHC class II expression within a population of infected cells, clones were derived and the levels of MHC class II expression within these discrete populations investigated. Exact quantitation of MHC class II expression was carried out using saturation binding analysis. The levels of MHC class II expressed by the different populations was then related to T cell stimulatory ability.

Results.

Section 3.2.

Characterisation of uninfected CD14$^+$ monocytes.

After MACS isolation of the CD14$^+$ population from PBM, aliquots of cells from the initial unfractionated population, the second CD14$^-$ population and the final CD14$^+$ population were stained with anti mouse Ig (whole molecule) FITC conjugate (binding to any VPM65 present). The purity of the resulting populations were assessed by FC analysis. Fig 3.1a shows an FC dotplot of the initial PBM population prior to MACS purification. This FC plot of forward scatter (FSC) vs side scatter (SSC) gives an indication of the size and internal complexity of the PBM population.

The majority of the cells contained in PBM are small and of low internal complexity, as signified by the low FSC and SSC shown by the main population. The cells showing higher FSCs and SSCs are larger more complex cells which are either cells of the myeloid lineage (ie, monocytes) or possibly small numbers of blasting lymphocytes (these cells are also larger than the resting PBM). The following dotplots of Log (FL1) fluorescence vs FSC (Fig 3.1 b-d) show the MACSed populations after incubation with FITC conjugate. Figs. 3.1b and c result from the staining of the

CD14 negative populations obtained subsequent to passage through the MACS column. Fig 3.1b shows VPM65 (CD14) staining of the initial negative population, whilst Fig 3.1c shows staining of the negative population following the second separation. Both of these dotplots contain very few positively labelled cells. Fig. 3.1d shows a predominantly VPM65 positive population (with approximately 90% of population positive for CD14).

Section 3.3.

Controls used during flow cytometry.

During FC analysis of macroschizont infected cells, negative control samples were run along side samples specifically labelled with primary mAbs and secondary conjugates. This allowed assessment of the extent of "nonspecific" binding of mouse immunoglobulins to the cells being assayed.

Negative controls for samples labelled with murine mAbs consisted of cells incubated on ice with normal mouse serum, at a dilution of 1:1500, followed by staining with the secondary conjugate. The use of normal sera was necessitated due to the lack of suitable isotype controls. Immunoglobulins contained in these sera act as a control for nonspecific binding of mouse/rat immunoglobulins to bovine cells. Comparison of negative control FC profiles to profiles produced after labelling with specific mAbs allows the true level of binding of specific mAbs to be ascertained.

FC analysis of T.a 12929 and clonal lines.

Once macroschizont infected cell lines had been established from the $CD14^+$ population, aliquots of one of these infected lines (T.a 12929) were soft agar cloned (as outlined in Chapter II). Of the fourteen clonal cultures isolated three were chosen for extensive study following preliminary FC analysis of MHC class II expression. The four populations studied were T.a 12929 (uncloned parental line) and clones G, I and L. All four macroschizont infected populations were analysed at the same time three weeks after cloning.

Fig. 3.1 **Flow cytometric analysis of $CD14^-$ and $CD14^+$ cells before, during and after MACS sorting.**

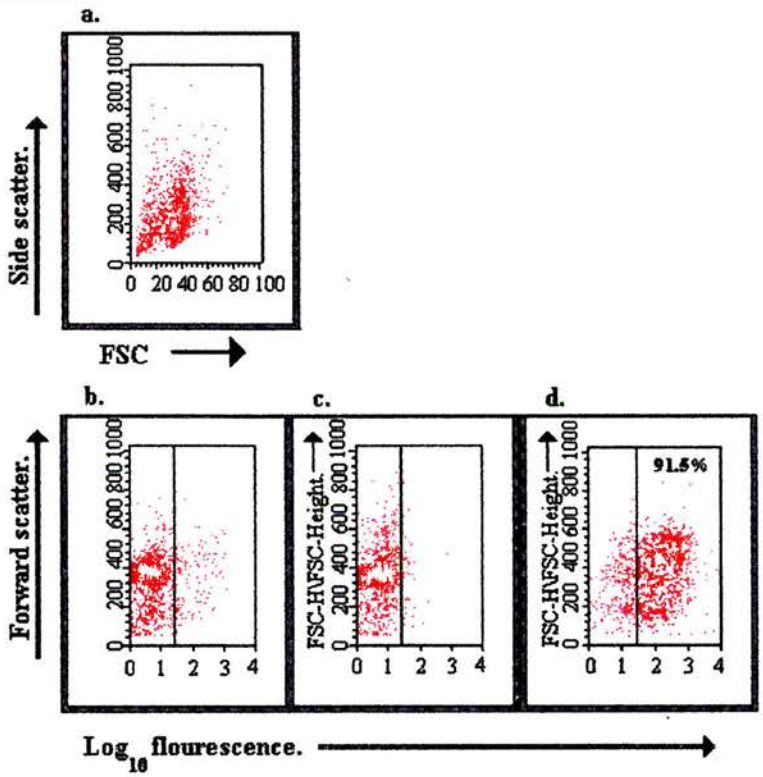
(a) Flow cytometry dotplot showing forward scatter (FSC) against side scatter (SSC) of whole PBM before MACS separation of $CD14^+$ cells, (b) dotplot of unseparated PBM population, stained with VPM65 showing $\log_{10}$ florescence against FSC, (c) dotplot ($\log_{10}$ florescence against FSC) of the $CD14^-$ population after the second MACS separation and (d) dotplot of final $CD14^+$ population after two MACS purifications ($\log_{10}$ florescence v.s. FSC), $CD14^+$ cells are shown in the gated region to the right.

Relative staining intensities of cell populations at stages through the $CD14^+$ cell purification.

Data shown as mean fluorescence.

(a) Whole unseperated PBM (unstained)	=	5.51.
(b) Whole PBM stained with VPM65	=	6.51.
(c) $CD14^-$ population after second MACS separation	=	6.05.
(d) $CD14^+$ population after separation stained with VPM65	=	115.22.

Fig. 3.1


a.
Side scatter.
FSC
0 20 40 60 80 100
0 200 400 600 800 1000
b.
c.
d.
Forward scatter.
FSC-H\FSC-Height
91.5%
0 1 2 3 4
Log10 flourescence.

Flow cytometric (FC) data produced during this investigation was used to compare the expression of various molecules by the parental and clonal populations of infected cells. Comparisons were based on the variation in fluorescence shown by the different samples following staining with a range of mAbs/fluorescent conjugate. The mAbs chosen for use in this part of the study are listed in Chapter II and were chosen either to highlight the expression of molecules associated with monocytes/MΘs or to show the lack of expression of markers for other cells such as CD3 (a T cell marker).

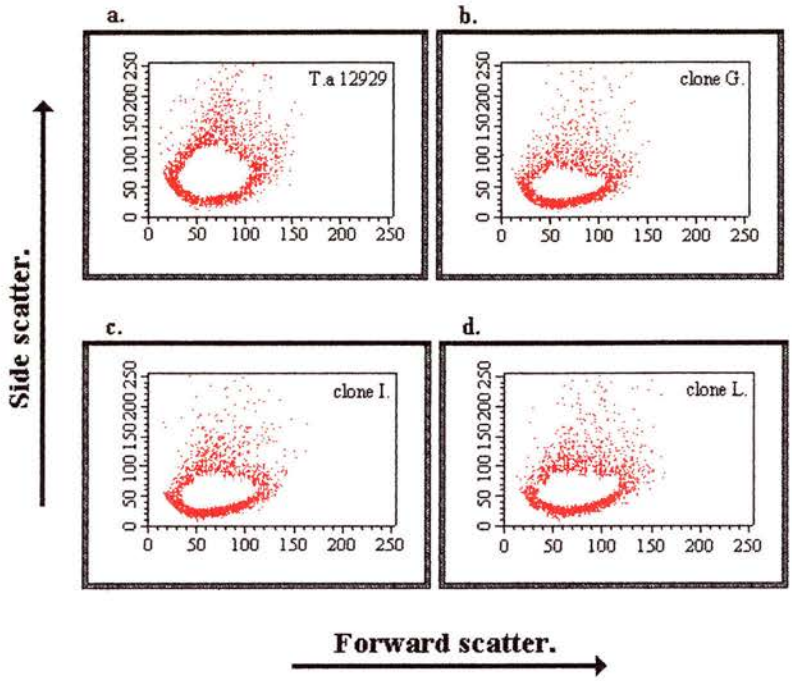
Figures 3.2 to 3.6 show the size/internal complexity and expression of various molecules on the surface of the parent population (T.a 12929) and the clonal lines derived from this population (clones G, I and L). The size and internal complexity of the cells within these different cell lines are all similar (Fig 3.2 a-d), and are characteristic of cell lines produced by infection with *T.annulata* (Campbell *et al*, 1994). After *in vitro* infection of $CD14^+$ cells with *T. annulata* sporozoites, the cells rapidly assume a large size (high forward scatter (FSC)), this can be seen if Fig. 3.1 and Fig 3.2 (a-d) are compared. Figs 3.2 (a-d) also show the parent and clonal lines contain cells of differing levels of internal complexity (*e.g.* mean FSC of PBM = 30, mean FSC of infected cells = 75).

As the size of the cells alters so does the expression of various surface molecules. These molecules include MHC class I and class II, CD14 and the epitope bound by the I.L.R.I. mAb IL-A24 (associated with antigen presentation and a mature MΘ phenotype) (Ellis *et al*, 1987; Campbell *et al*, 1994). Infection of cells with this parasite has markedly different effects on the expression of the two forms of MHC molecules expressed on the cell surface. The expression of MHC class I (labelled with the mAb IL-A19) by cells of the parent and clonal populations are very similar (Fig. 3.3 a-d), with all the cells of the four populations highly MHC class I positive. Also the range of expression of MHC class I within these populations are low, shown by the narrowness of the normally distributed positive histograms.

Fig. 3.2 **Flow cytometry dotplots of T.a 12929 and clonal populations.**

(a-d) Flow cytometry dotplots of T.a 12929 and clones G, I and L respectively, showing FSC against SSC. The four infected cell populations can be seen to be similar in size (forward scatter) and internal complexity (side scatter).

Fig. 3.2


a.
b.
c.
d.
T.a 12929
clone G.
clone I.
clone L.
0 50 100 150 200 250
Side scatter.
Forward scatter.

This is very different from the expression of MHC class II molecules by the T.a 12929 cell line and the clones derived from it. These populations express varying levels of MHC class II molecules, a phenomenon which has been observed before during analysis of numerous other unrelated *T.annulata* infected cell lines (Campbell, pers. comm.). In contrast to the narrow unimodal expression of MHC class I by the parent line and clones, the MHC class II expression varies greatly between clones and parent line and also shows variability within the individual cell populations (Fig 3.3 e-h). It can be seen that the parental population (T.a 12929) shows a broad expression of MHC class II molecules, whereas the range of MHC class II expression on the clonal populations appears narrower. Clone G appears to express the highest levels of MHC class II molecules of the three clones, whilst clones I and L both express lower levels of these molecules.

The levels of the two MHC class II molecules expressed on bovine cells were assessed by staining with the mAbs VPM36 (binding MHC class II DQ) and VPM54 (binding MHC class II DR) (Fig 3.4 a-d and e-h respectively) (Dutia *et al*, 1993; Dutia *et al*, 1995). Analysis of the FC data obtained from staining of the parent line and clones with these two mAbs (Fig. 3.4) shows that both these molecules are expressed at similar levels within each population and that the variation in the levels of MHC class II expression is not solely due to the higher expression of one or other MHC class II molecule.

Following the infection of cells by the parasite, expression of the LPS receptor (CD14) decreases dramatically. Staining of the parent and clones with the mAb VPM65 after infection with sporozoites shows that these cell lines do not express CD14 (Fig 3.5 a-d). The cells used to produce these lines were selected on the basis of CD14 expression, with infectivity of the $CD14^{+}$ populations shown to be markedly higher than non-CD14 expressing populations (Campbell *et al*, 1994). However, after these populations became infected and formed established lines the expression of CD14 decreased dramatically. These data showing that infection of monocytes and macrophages with the parasite induces the loss of CD14 expression. The possibility that the parasite may have infected and "transformed" residual T cells in the purified population was assessed by staining the parent and clonal lines with the anti-CD3

mAb MM1A (Davis *et al*, 1993) (Figs 3.5 (e-h)). It can be seen that the expression of CD3 by the infected cells is effectively zero, suggesting that T cells are not suitable precursors for the production of *T.annulata* cell lines *in vitro*. Since the original population is highly $CD14^+$ (at approximately 90% positive) and taking into consideration the infectivity data of Spooner *et al* (1989), Innes *et al* (1992) and Campbell *et al* (1994), it seems highly unlikely that the parent and clonal lines contain T cells transformed with *T.annulata*.

Fig. 3.3

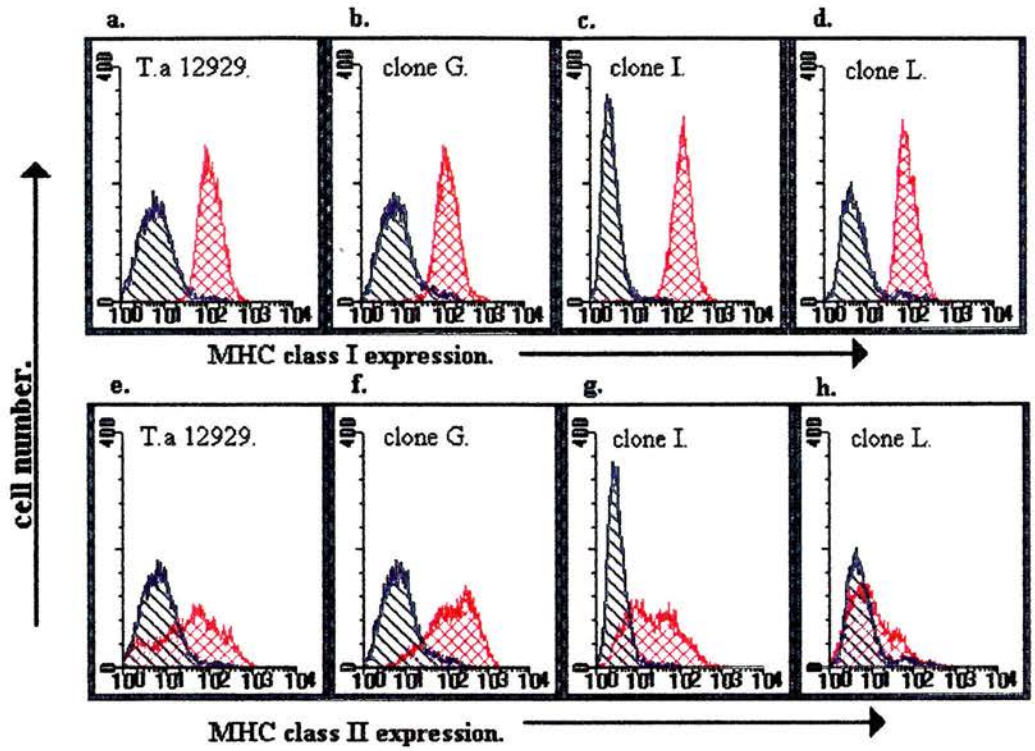

a.
T.a 12929.
b.
clone G.
c.
clone I.
d.
clone L.
MHC class I expression.
cell number.
e.
T.a 12929.
f.
clone G.
g.
clone I.
h.
clone L.
MHC class II expression.

Fig. 3.4 **Flow cytometric data relating to the expression of the two MHC class II molecules DQ and DR by T.a 12929 and clonal populations G, I and L.**

(a-d) MHC class II DQ expression of T.a 12929 and clones G, I and L. Blue profiles represent the negative control samples, whilst red represent samples labelled with the anti MHC class II DQ mAb VPM36. (e-h) MHC class II DR expression of T.a 12929 and clones G, I and L. Blue profiles represent negative control samples and red represent samples labelled with the anti MHC class II DR mAb VPM54.

Fig. 3.4

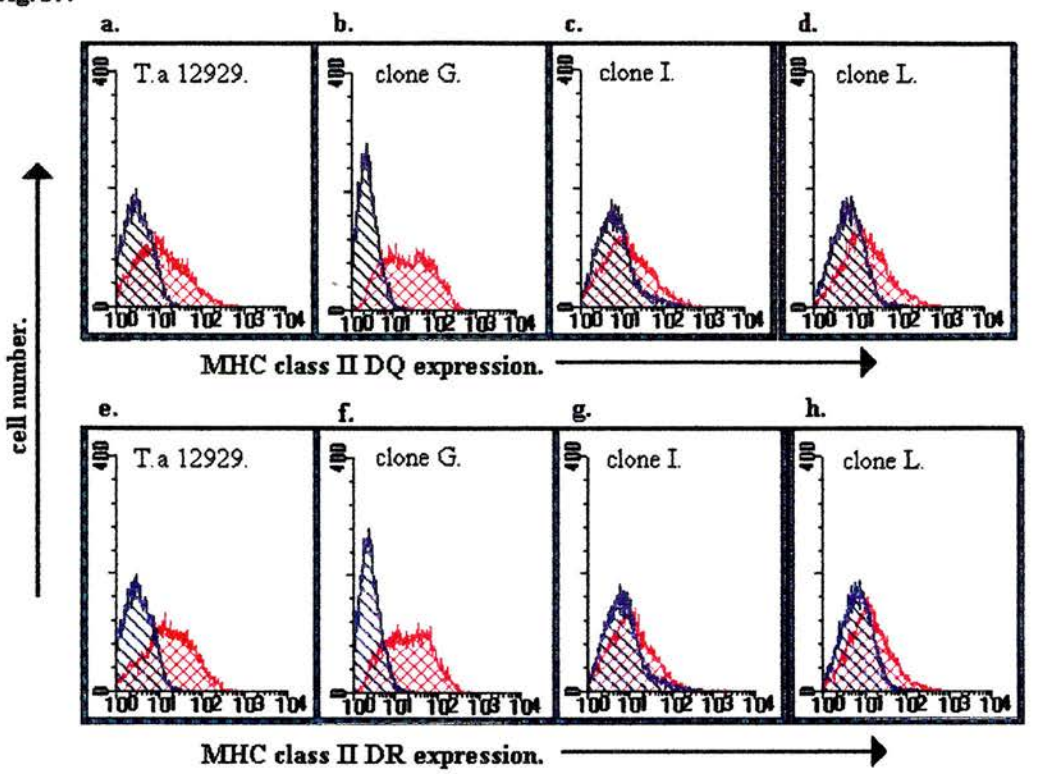
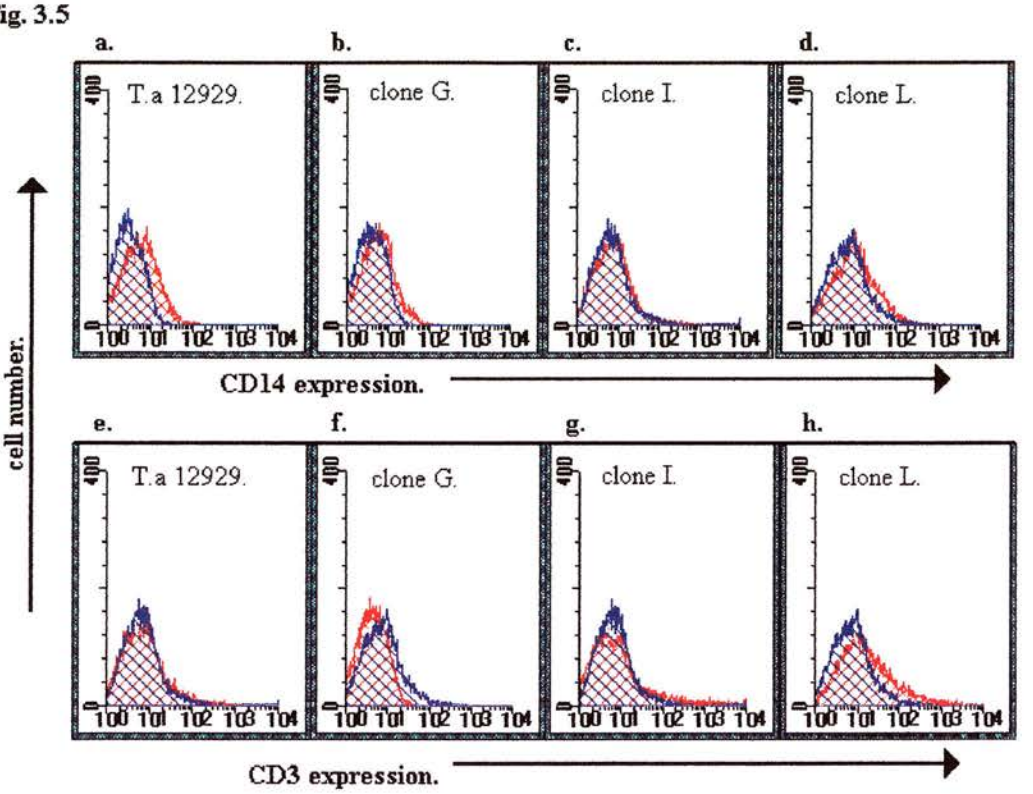

a.
b.
c.
d.
T.a 12929.
clone G.
clone I.
clone L.
cell number.
MHC class II DQ expression.
e.
f.
g.
h.
T.a 12929.
clone G.
clone I.
clone L.
MHC class II DR expression.

Fig. 3.5 **FC data relating to the expression of the surface molecules CD14 and CD3 by infected cell populations, T.a 12929 and clones G, I and L.**

(a-d) Expression of CD14 (the LPS receptor) by parent and clonal lines (G, I and L). Negative control profiles are shown in blue, samples labelled with the anti CD14 mAb VPM65 are shown in red. (e-h) Expression of the T cell marker CD3 by the same four populations. Negative control samples are blue, samples labelled with the anti CD3 mAb are red.

Fig. 3.5

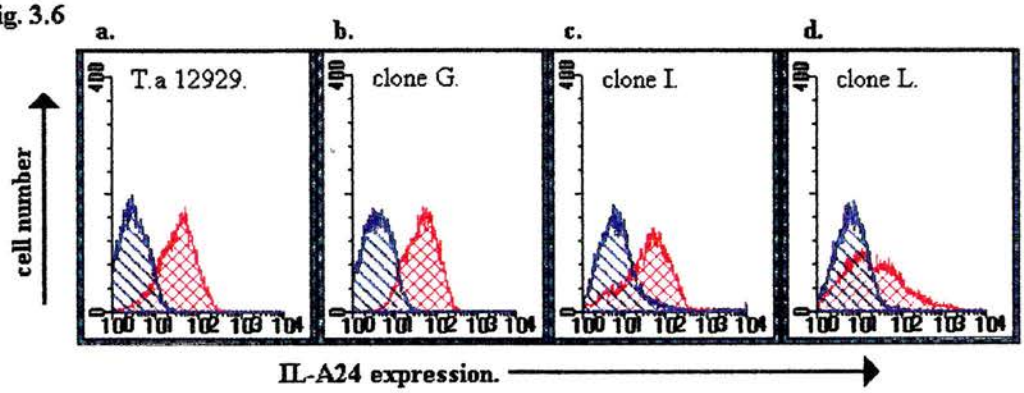

a.
b.
c.
d.
T.a 12929.
clone G.
clone I.
clone L.
CD14 expression.
e.
f.
g.
h.
T.a 12929.
clone G.
clone I.
clone L.
cell number.
CD3 expression.

The mAb IL-A24 was used to investigate the expression of an epitope associated with monocytes and MΘs (Ellis *et al*, 1987) by T.a 12929 and the clonal lines. Previous work has shown this epitope to be expressed on the $CD14^+$ populations isolated from PBM by MACS purification (Campbell *et al*, 1994). The epitope is also expressed to varying degrees by *T.annulata* infected cell lines (Campbell *et al*, 1994; Campbell, 1995). The parental line and the clones express the epitope bound by IL-A24 but the expression varies between these lines (Fig 3.6 a-d). Expression of this epitope by the parental line and clones G and I, showed a smaller range of expression than clone L, depicted by the broad peak seen in Fig 3.6d as opposed to those shown in Fig 3.6 a-c.

Fig. 3.6 **Flow cytometry data relating to the expression of a macrophage associated antigen by the infected cell populations T.a 12929 and clones G, I and L.**

(a-d) Expression of the macrophage associated epitope bound by the mAb IL-A24, by macroschizont infected cells of T.a 12929 and clones G, I and L. Negative control profiles are shown in blue and profiles of cells labelled with IL-A24 are shown in red.

Fig. 3.6


a.
b.
c.
d.
T.a 12929.
clone G.
clone I.
clone L.
400
0
100 101 102 103 104
cell number
IL-A24 expression.

Section 3.4

Saturation binding studies using ^{125}I-SW73.2.

Table 3.1

Cell line.	Mean No. of MHC class II molecules/cell.	Range
Uninfected CD14$^+$ cells.	$2.65x10^5$ (S.D. ± $4.6x10^4$)	$2.32x10^5$- $2.98x10^5$
clone I.	$8.99x10^4$ (S.D. ± $8.2x10^3$)	$8.00x10^4$ - $1.00x10^5$
clone L.	$1.52x10^5$ (S.D. ± $4.5x10^4$)	$1.00x10^5$ - $2.00x10^5$
clone G.	$1.16x10^6$ (S.D. ± $7.7x10^5$)	$1.00x10^6$ - $2.00x10^6$
T.a 12929.	$2.40x10^6$ (S.D. ± $9.6x10^4$)	$2.40x10^6$ - $2.50x10^6$
BL20 cell line.	$2.50x10^5$ (S.D. ± $6.8x10^4$)	$2.02x10^5$ - $2.98x10^5$

Table 3.1 MHC class II cell surface expression on T.a 12929 and cloned cell lines determined by saturation binding studies.

Data produced during saturation binding analysis using ^{125}Iodine labelled SW73.2 A(ab')$_2$ fragments. Aliquots of $1x10^6$ cells were incubated with radiolabelled SW73.2 on ice for 30 mins. Unbound label was washed from the sample and the amount of label bound assessed by gamma counting. The average number of MHC class II molecules expressed per cell were then calculated. The standard deviations shown represents variation of data between separate SW73.2 saturation binding assays.

Table 3.1 shows data from the saturation binding studies in which triplicate aliquots of $1x10^6$ cells of each cell line or clone were incubated with ^{125}I-SW73.2 under saturating conditions. The mean number of MHC class II molecules expressed per cell (Table 3.1) were determined from four independent experiments (except for the BL20 and uninfected CD14$^+$ cells for which data are shown from two experiments). The

parent cell line expressed higher levels of MHC class II than any of the clonal populations. Clone G showed the highest expression of MHC class II of the clones, whilst clones I and clone L expressed similarly low levels of MHC class II. The BL20 cell line was included in the analysis to assess the efficiency of the saturation binding technique, as this cell line has been shown to express approximately $2x10^5$ MHC class II molecules per cell (Hopkins *et al*, 1989)

Statistical analysis (using the Student t-test), showed variation in MHC class II expression within triplicate samples of the parent line and clones not to be statistically significant. However, the expression of MHC class II by the parent line and clone G (although not statistically different from each other) was shown to be significantly different from clones I, L and uninfected cells ($P \leq 0.001$). The MHC class II expression by clone I was not significantly different from that of clone L. These experiments were conducted over a period of 13 months, during which time the variation of MHC class II expression by the parental and clonal lines was minimal (as shown by the standard deviations of MHC class II expression by the parent and clonal lines in Table 3.1).

Section 3.5.

Assessment of T cell proliferation induced by contact with infected cells.

To optimise T cell proliferation experiments and investigate the kinetics of T cell proliferation, infected cells (of either T.a 12929 or the clonal cultures) were cultured with autologous PBM for up to seven days (Fig. 3.7). This assay was designed to ascertain whether the day of peak proliferation did occur on day 5 (as shown by Campbell (1995)) and from the graph it can be seen that this is the case.

The data is shown as corrected cpm (ccpm), corrected cpm = to: the cpm of the proliferating cells minus the cpm of the infected cell control and the cpm of the PBM control for each particular day. The data shows that the presence of infected cells does not induce perceptible proliferation until day 3. From this day all the PBM cells stimulated begin to proliferate, except those incubated with clone G, which only begins to exhibit a positive net proliferation on day 5. It is at this point that the proliferation of all PBM samples peak, with clone L showing the highest level of

proliferation (at ccpm = 62031.4) and clone I the lowest (at ccpm = 4702.5). From day 6 the amounts of ^{3}H-thymidine incorporated into all the PBM samples begin to decline, regardless of the infected cells used to stimulate the T cells present. At day 7 the proliferation of T cells has subsided in all cultures, with only T cells incubated with the parent line showing a small amount of proliferation. These data agreeing with the findings of Campbell (1995).

Fig. 3.8 shows the T cell proliferation induced in subsequent experiments, when autologous PBM were incubated for 5 days in the presence of T.a 12929 or clones I, G or L (data shown from four repeat experiment). Clone L induced the highest net levels of proliferation (44066ccpm ± 4504), which was significantly higher than the proliferation induced by the parent line and clone I ($P \leq 0.005$). Both clone G and the parent line induced similar levels of proliferation, with both cell lines inducing levels of proliferation lower than clone L (22994ccpm ± 7565; 16636ccpm ± 6657 respectively). Whilst in contrast, clone I induced extremely low proliferation (5727ccpm ± 1274), significantly lower than all other infected cells ($P \leq 0.005$).

Fig. 3.7 **Six day time course experiment showing T cell proliferation induced by autologous infected cell populations T.a 12929 and clones G, I & L.**

Data collected from a time course experiment investigating T cell proliferation when PBM were incubated/stimulated with either mitomycin C treated cells of T.a 12929 or one of the clonal cultures (G, I or L), on days 2 to 7 after macroschizont infected cells are added to PBM at a ratio of 1:10. Data is presented as the amount of tritiated thymidine incorporated into proliferating T cells (shown as corrected cpm) on days 2 to 7. Counts obtained from samples of infected cells and PBM incubated separately over the 5 day time course were subtracted from those measured for each proliferating population of infected cells plus PBM. The figure produced is the amount of T cell proliferation induced by the presence of each infected cell population.

Uncorrected cpm of samples on day 5 of incubation (data shown as mean values calculated from four replicate samples).

PBM alone= 4834.4cpm.

T.a 12929 cells= 13326.8cpm.	T.a 12929+PBM = 28614.1cpm.
clone G cells= 10814.3cpm.	clone G+PBM = 29005.0cpm.
clone I cells= 8526.6cpm.	clone I+PBM = 18063.5cpm.
clone L cells = 4462.6cpm.	clone L+PBM = 71328.0cpm.

Fig. 3.7

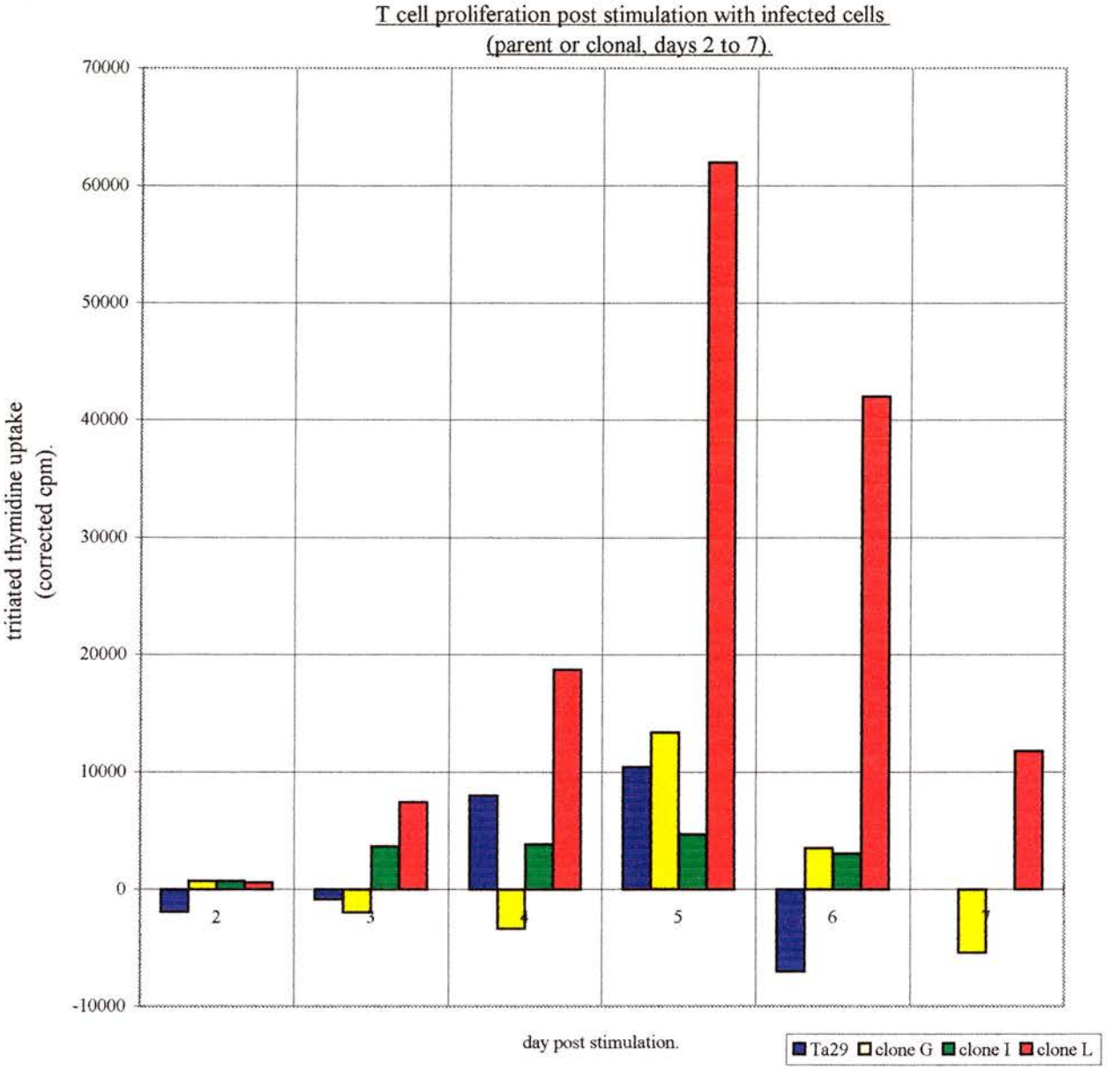
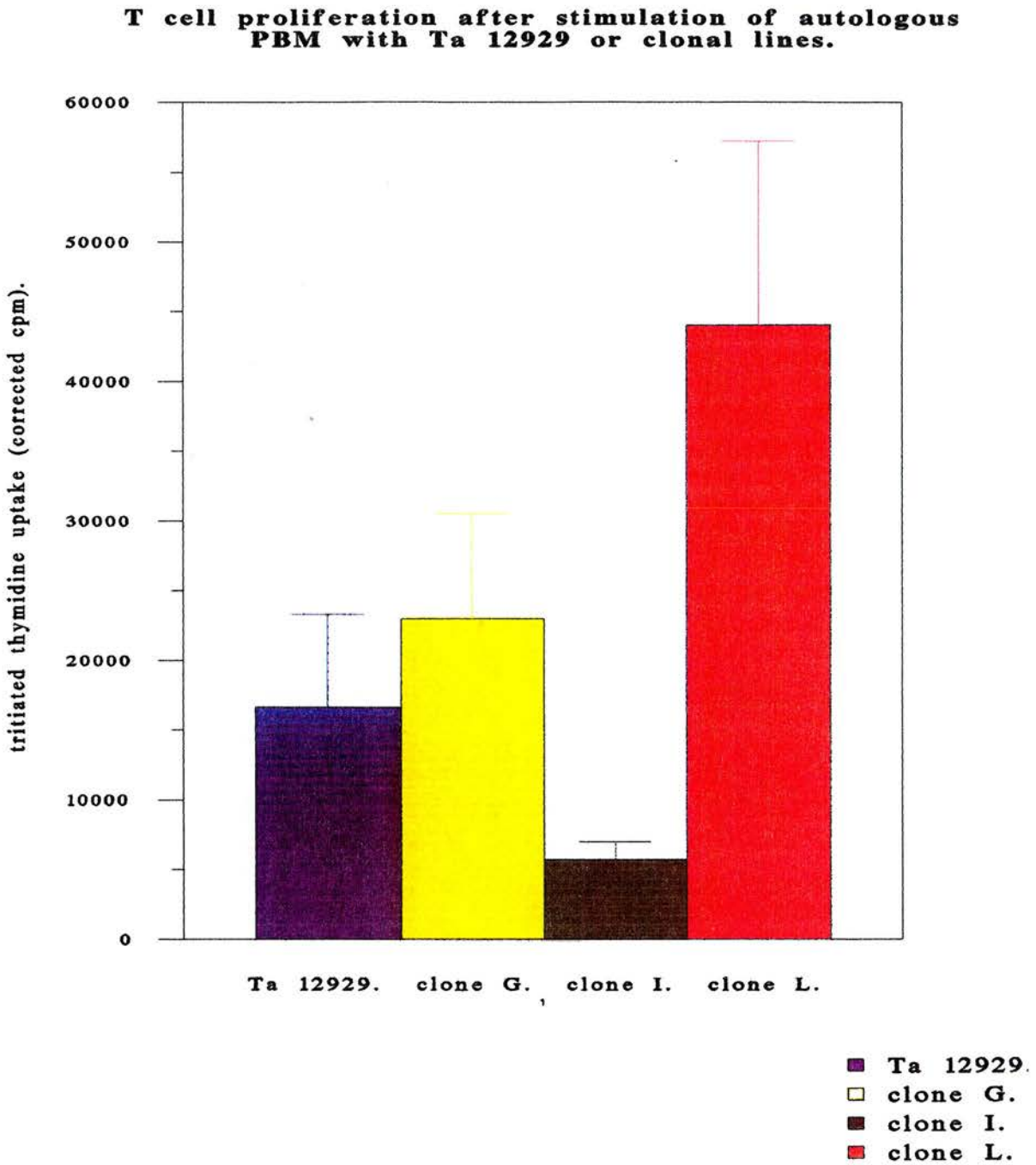

T cell proliferation post stimulation with infected cells
(parent or clonal, days 2 to 7).
tritiated thymidine uptake
(corrected cpm).
70000
60000
50000
40000
30000
20000
10000
0
-10000
2
3
4
5
6
7
day post stimulation.
Ta29
clone G
clone I
clone L

Fig. 3.8 **T cell proliferation induced after autologous PBM were incubated with infected cells of either T.a 12929 or clones G, I and L for five days.**

T cell proliferation data from four repeat experiments. PBM were incubated with mitomycin C treated infected cells of the parent line or clones (G, I or L) at a ratio of 10:1 and harvested on day 5. Data is shown as corrected cpm of tritiated thymidine uptake by T cells proliferating following stimulation with either the parent line or a clonal population (error bars shown represent standard deviations over the four experiments).

Fig. 3.8.


T cell proliferation after stimulation of autologous PBM with Ta 12929 or clonal lines.
tritiated thymidine uptake (corrected cpm).
60000
50000
40000
30000
20000
10000
0
Ta 12929.
clone G.
clone I.
clone L.
Ta 12929.
clone G.
clone I.
clone L.

Discussion.

A highly purified population of $CD14^+$ monocytes and MΘs were used to produce the *in vitro* infected lines, ensuring infected cells were of the monocyte/MΘ lineage, shown by Forsythe *et al* (1997) to be infectable *in vivo*. The establishment and subsequent cloning of these lines permitted investigation into the expression of a number of cell surface markers (*i.e.* MHC class I/II molecules), without encountering high levels of variation in expression, due to the heterogeneous nature of infected cell populations derived after infection of whole PBM. The main area of study described in this chapter investigated the *in vitro* expression of MHC class II molecules by macroschizont infected cells and more briefly the expression of MHC class I molecules.

Alterations in MHC molecule expression. - (1) MHC class II molecules.

Initial work by Dr E. Glass showed the *T.annulata* infected cell lines expressed high levels of MHC class II and it was suggested that this may be linked to the ability of macroschizont infected cells to cause high levels of proliferation, in cultures of naive autologous T cells (Glass & Spooner, 1990). The work shown in this chapter suggests that the presence of the parasite within infected cells does not simply result in an upregulation of MHC class II expression but that the expression of these molecules undergoes a "dysregulation" following infection.

MHC class II expression is upregulated following activation of monocytes and MΘs (Keller, *et al*, 1994), allowing the Ag presenting cells to interact with T cells and form a suitable immune response. As is shown by the saturation binding assay data concerning the expression of MHC class II by uninfected monocytes and MΘs, expression prior to infection is low. However, after infection with *T.annulata* the expression of MHC class II molecules alters, with the majority of cultures exhibiting an increase in expression. The changes induced by infection do not mimic those post activation (Keller, *et al*, 1994), as class II expression does not simply rise. This can be seen from the data of class II expression, which show that within the parent population (T.a 12929) there are populations (which can be cloned out) that do not

express high levels of class II after infection.

It was also found that the expression of MHC class II by the parental line (T.a 12929) and the clonal lines was relatively constant. The populations investigated showed very little variation with respect to MHC class II expression during the months over which these cells were studied. The upregulation of MHC class II expression by uninfected professional APCs is generally transient (Unanue & Allen, 1987), with the expression decreasing after the immunological reactions have peaked. In contrast the expression of MHC class II by the *T.annulata* infected cells studied does not vary, suggesting that the alterations in MHC class II expression are long lasting and possibly permanent following infection.

(2) MHC class I molecules.

The constant expression of MHC class II is paralleled by the constant expression of MHC class I molecules by infected cells. MHC class I molecules are constitutively expressed by the majority of mammalian cells (exceptions including, red blood cells and cells of the central nervous system) and are involved in the recognition of endogenous antigen in cells infected with intracellular pathogens. However, the range of expression of MHC class I by *T.annulata* infected cells differs greatly from the expression of MHC class II molecules. Data from this chapter shows MHC class I expression to be very similar in all the populations investigated. The expression of MHC class I by uninfected cells was not investigated, therefore it is not possible to compare the expression of MHC class I before and after infection. However, investigation of the expression of MHC class I molecules by FC analysis shows the infected populations express very similar levels of these molecules, when stained with the anti MHC class I mAb IL-A19. The peaks shown on the resulting histograms are of a uniform shape and the degree of fluorescence is also similar.

Differences in class I and class II expression by infected cells.

The data concerning MHC class I expression differ markedly from that obtained for MHC class II expression. The quantitation of MHC class II expression by the parental line and the clones yields the mean number of MHC class II molecules expressed per

cell, within the whole population. On the other hand the non quantitative FC analysis shows that within the populations the level of expression of MHC class I is much more uniform than the expression of MHC class II. The data showing that cells within these populations express similar levels of MHC class I molecules but that there is a wider range of MHC class II expression.

There are a number of reasons which could account for these differences, *e.g.* the expression of MHC class I is not altered by infection or that the parasite does alter expression of these molecules, producing uniform expression within a population. For the parasite to produce such similar MHC class I expression between four populations and also within other populations (produced from different parasite stocks and from different animals) also investigated in our laboratory (J. D. M Campbell, pers. comm. (data not shown)) would seem unlikely. This suggests that the MHC class I expression observed is similar to that of uninfected cells. However, further work need to be carried out in this area, with saturation binding techniques being applied to MHC class I quantitation.

The data shown in this chapter suggests that the control of class I expression is different from that of class II post infection. The parental population which gave rise to the clonal cultures used in this investigation has a broad range of expression of MHC class II. The spread of expression by the clonal cultures appears to be less than the original population and this would seem logical, since each population is derived from a single infected cell. However, the mechanisms by which the average level of MHC class II expression approximately constant are unknown.

The expression of MHC class II varies within the parent population, the wide range of this variation presumably arises in part from the fact that many different cells will have become infected and expanded to form subpopulations within this non clonal line. If the main cause of variation with respect to MHC class II was the different cells which were infected by the parasite, then cloning should have greatly reduced the variation of MHC class II expression within the clonal cultures. This does not appear to be the case, with the clones still exhibiting variation in the levels of MHC class II expression. A possible explanation for the variation of MHC class II expression within infected cultures is that the levels of MHC class II expressed by

cells may vary within different stages of the cell cycle, *i.e.* newly formed macroschizont infected daughter cells may express lower levels of MHC class II than do more phenotypically mature infected cells. FC analysis of MHC class II expression within cell cycle synchronous cultures, studying variations in expression levels as the cultures mature and proliferate could explain why clonal cultures still express a wide range of MHC class II.

Another possible reason for differences in class II expression could be that a single cell can play host to more than one parasite. Work has shown that multiple infections of single cells are possible and that cells containing parasite derived from more than one sporozoite are viable (Jura, 1986). If this is the case it could be that differing parasites within the clonal cultures are able to influence the range of expression of MHC class II. This would suggest that during the expansion of the clonal population individual parasites behave differently to cause variations in MHC class II expression. The actual importance of the roles played by the parasite and the cell with respect to the expression of MHC class II are not understood and there are conceivably many ways in which the parasite could interfere with MHC class II expression. These could include effects such as the production of molecules by the parasite which interfere with transcriptional or translational regulation, thus altering MHC class II expression. Whatever the mechanism employed by the parasite to alter class II expression it is reversible, as treatment with theilericidal drugs returns infected cells to a non proliferating, uninfected state (McHardy *et al*, 1984) (Rintelen *et al*, 1990).

Another possibility which exists is that infected cells are capable of inducing the production of molecules which are stimulatory for cells of the monocyte/Mθ lineage. Nichani (1994) showed that during clinical cases of tropical theileriosis very high levels of IFNγ were detectable in efferent lymph. IFNγ is a cytokine messenger produced by activated T cells (generally of the Th_1 type) which has powerful effects on monocytes and Mθs (Ijzermans and Marquet, 1989; Mogensen *et al*, 1987). Also high levels of IFNγ mRNA have been detected within autologous *T.annulata* stimulated T cells (Campbell, 1995). MHC class II expression is known to be upregulated by treatment of macrophages with this and other cytokines. This could mean that the expression of MHC class II molecules by infected cells is effected by

levels of cytokines present in their environment. Investigation of the production of cytokines by infected cells are discussed in Chapter IV.

The changes observed in the expression of MHC class II molecules by infected cells also appear to affect the DR and DQ class II products equally. The data concerning this phenomenon is not quantitative, however, this is suggested by the similarities between FC profiles produced by labelling with the mAbs VPM36 (DQ specific) and VPM 54 (DR specific). These data suggest that interference of the parasite in the expression of MHC class II molecules is not locus specific and that the whole MHC class II region is targeted.

It has been suggested that the "nonspecific" proliferation induced by contact of macroschizont infected cells with autologous $CD4^+$ T cells may occur through the activity of a superantigen like molecule associated with MHC class II molecules (Campbell, 1995). However, the data contained in this chapter shows that the levels of MHC class II expression do not correlate to the levels of T cell proliferation induced by infected cells. Since T cell activation by infected cells has been shown to be contact specific it may be that infected cells do possess a superantigen like activity. However, I suggest that this is not the only factor involved in autologous T cell activation.

CD14 expression and APC function of infected cells.

CD14 is the glycophosphatidylinostitol linked surface receptor for bacterial lippopolysaccheride, which is expressed on the surface of monocytes, MΘs and a small population of B cells (Goyert *et al*, 1988; Zeigler-Heitbrock & Ulevitch, 1993; Yang *et al*, 1995). This receptor binds gram negative bacterial LPS, associated with LPS binding protein (LBP) and initiates cellular activation (Wright *et al*, 1990) (Ulevitch *et al*, 1994). The expression of CD14 by monocytes and MΘs is essential for their function within innate protection, with rapid activation and production of proinflammatory cytokines providing a first line of immunological defence. Activation of these cells via this receptor causes cells to increase their metabolic rate and produce detectable chemicals such as reactive oxygen intermediates (ROIs) (Emmendorffer *et al*, 1990), which are necessary to destroy invading microorganisms following

opsonisation and endocytosis.

CD14 has also been implicated in a negative feed back pathway which can inhibit monocyte dependent T cell proliferation (Lue *et al*, 1991). Engagement of human CD14 (by an anti CD14 mAb) prior to incubation with T cells appears to induce a signal which is able to inhibit T cell proliferation. The signal strength appears to be sufficient to inhibit PHA or ionophore-induced proliferation. Studies performed by Grey *et al* (1994) show induction of TNF-α production, down-regulation of CD14, and down-regulation of the other LPS-binding proteins CD11b and CD18, following Mθ activation using either LPS or IFNγ treatment. Presumably this receptor is down-regulated after activation because the CD14 signal is no longer required. It is also possible that down-regulation of CD14 is necessary to control cytokine production from activated Mθs, *ie* to prevent over-stimulation of Mθs.

The loss of CD14 expression by *T.annulata* infected cells may aid the parasite by inhibiting negative feedback signals to T cells, allowing the *T.annulata* induced T cell proliferation to proceed, thus subverting cell mediated immune reactions. Another possibility is that the CD14 receptor is down-regulated to prohibit initiation of secondary signal cascades within the infected cells due to LPS+LBP/CD14 binding on the surface of infected cells. If legitimate signalling via CD14 were possible within infected cells, the intracellular reactions produced may prove deleterious for the parasite.

The epitope bound by IL-A24 is associated with APC function (Ellis *et al*, 1987) and data shown in Fig. 3.7 a-d shows that the macroschizont infected cell lines derived from $CD14^+$ cells, used in this study (T.a 12929 and clones G, I and L) express this molecule. A study by Campbell *et al* (1994) showed *T.annulata* macroschizont infected cell lines express this Mθ associated epitope. As noted macroschizont infected lines exhibit an augmented APC function (Glass and Spooner, 1990a). When these data are viewed together it suggests that the presence of macroschizonts within monocytes/Mθs alters APC function. Whether alterations in the APC function of these cells are specifically induced by the parasite or are an artefact following infection requires further study.

Changes in the growth/division of infected cells.

Macroschizont infected MΘ populations are unusual with respect to the fact that they are constantly proliferating. *In vivo* (Barnett, 1977; Melhorn and Schein, 1984) and *in vitro* (Hulliger *et al*, 1964) infected cells are seen to proliferate and the infected population to expand rapidly. This is also the case with the parent line and clonal populations involved in this study, with it being necessary to reduce infected cell cultures three times a week. The reduction of these cultures maintained the cells in a state of healthy rapid growth, necessary for comparative experiments to be performed (Campbell *et al*, 1994. This was presumably due to the loss of condition by the cells and build up of cellular detritus within the culture (which became visible at the bottom left hand corner of FC dotplots and could be gated out). However, it was observed that the expression of certain molecules (such as MHC class I and class II molecules) did not resemble that of freshly passaged cultures with peaks on the FC histograms becoming diffuse and irregular. This is again presumably due to the loss of condition of the infected cells.

These observations agree with work carried out in the Divisions of Veterinary Infectology and Immunology and Molecular Immunology, Borstel, Germany. Here Shayan showed that cells infected with *T.annulata* did not appear to possess a G_0 phase in their cell cycle (Shayan *et al*, 1994). During this investigation a mAb known as anti Ki-67 (Gerdes, *et al*, 1983) was used to investigate the expression of nuclear antigens associated with the active part of the cell cycle by *T.annulata* infected cells. Anti Ki-67 was originally used to observe the expression of proliferation associated antigens in human cells. It was found that this antigen was expressed in the G_1, S, G_2, and mitosis phases of the human cell cycle but was not expressed during the G_0 phase. Shayan investigated the expression of this antigen in macroschizont infected cells and found expression of an antigen which reacted with Ki-67. However, after treatment with the theilericidal drug, Buparvaquone (McHardy *et al*, 1985) expression of the antigen decreased and was sometimes absent. Buparvaquone treatment led to parasite death and the establishment of a G_0 phase within the cell cycle.

Subsequent comparison of cDNA clones showed that the antigen expressed by bovids showed a high degree of homology to human Ki-67. These data suggest that the

bovine and human Ki-67 antigens are similar. From the lack of a G_0 phase in the infected cells cell cycle, which fails to express the Ki-67 antigen we can surmise that infected cells do not express a G_0 phase but are constantly kept in the active phases of the cell cycle. Cells also appear to be in a state of permanent activation, suggested not only by the lack of $CD14^+$ (Grey *et al*, 1994) but also by the constant production of a range of cytokine mRNA species (which will be discussed in Chapter IV). This state of constant activation/proliferation may be linked to the lack of variation in the expression of surface markers such as MHC class I and II after infection (when the cells are maintained in a healthy condition).

The reason why *T.annulata* should cause alterations in the expression of MHC class II is not known as yet. However, it may be that the dysregulation of MHC class II expression is a mechanism for the general disruption of the immune system, allowing the parasite to multiply within infected cells without interference by the hosts immune system. It is known that infected cells have the ability to stimulate proliferation of both naive (Glass & Spooner, 1990) and memory T cells (Campbell, 1995). Non-specific stimulation of these two subpopulations appears to lead to a T cell activation state which is ineffectual with respect to T cell help. Quantitating the levels of class II expressed by a number of cell lines meant that possible correlations between MHC class II expression and the non-specific T cell stimulatory ability of *T.annulata* infected cells could be studied.

T cell proliferation analysis.

The data contained within this chapter (section 3.5) shows that the ability to induce autologous T cells to proliferate varies depending upon the infected cell population used to stimulate the T cells, and although contact dependent (Campbell *et al*, 1995), does not correlate with the levels of MHC class II expressed on the surface of macroschizont infected cells. Also data presented in this chapter, show that infected clonal cultures derived from $CD14^+$ cells, stimulated similar kinetics of T cell proliferation (proliferation peaking on day 5 (Fig. 3.7)) in autologous T cells, as did infected cell lines produced by infecting whole PBM (Campbell, 1995).

As shown in Fig. 3.8, clone I induced very low levels of proliferation when compared to that induced by any of the other infected cultures. Also that the parent line was unable to induce the same levels of proliferation as clones G or L. The reasons for this phenomenon are not clear, however, it can be seen with respect to the data presented in Table 3.1 and Fig. 3.8, that the levels of proliferation induced do not correlate with the levels of MHC class II expressed by the stimulating cells. An infected cell line expressing over 10 times more MHC class II (clone G, $1.58x10^6$ molecules $cell^{-1}$) than another cell line (clone L, $1.44x10^5$ molecules $cell^{-1}$) reproducibly induced lower levels of T cell proliferation. This is again evident when one considers the level of MHC class II expression of the uncloned parent line (T.a 12929), which expressed $2.45x10^6$ molecules $cell^{-1}$ and that of clone G (clone G, $1.58x10^6$ molecules $cell^{-1}$). Clone G expresses lower levels of class II but still induces larger amounts of T cell proliferation. Clone I differs from all of these cell lines, as this population of infected cells expresses the lowest levels of class II and also induces the lowest levels of T cell proliferation.

This data does not however, necessarily discount a role for MHC class II in the activation of T cells by infected cells, as it has been shown by many groups that MHC class II molecules play an integral role in T cell activation. (Unanue, 1984) and as noted T cell activation by *T.annulata* infected cells is contact dependent (Campbell *et al*, 1995). To test this hypothesis one would need *T.annulata* infected lines/clones devoid of class II expression, which would allow the role played by class II expression during T cell activation to be further dissected.

After these studies detailed above had been completed a supply of the anti MHC class II mAb IL-A21 became available and during blocking studies of T cell activation by *T.annulata* infected cells it was found that, blocking TCR/MHC class II interactions prohibited T cell activation/proliferation (J. D. M. Campbell, pers. comm.). This suggests that although the actual numbers of MHC class II molecules expressed by infected cells may not correlate with the levels of T cell proliferation induced, MHC class II molecules may play a role in "non-specific" T cell activation by macroschizont infected cells. However, it may be that as long as there is a basal level of MHC class II molecules expressed by infected cells, they will possess the ability to induce a

certain level of "nonspecific" T cell proliferation.

The mechanisms which are inducing these different levels of T cell proliferation are not known but one possible cause of this phenomenon could be the production of cytokines by macroschizont infected cells. It is known that monocytes and MΘs are capable of producing T cell stimulatory cytokines (such as, IL-1 (DeFreitas *et al*, 1983, Dower *et al*, 1992) and IL-6 (Navarro *et al*, 1989; Van Snick, 1990)). The possibility that the infected cells used during this study produce cytokines which then effect the proliferation of responding T cells was investigated and the findings are detailed in chapter IV.

Chapter IV.

Cytokine mRNA expression by *Theileria spp.* infected cell lines and correlation with autologous T cell proliferation.

Section 4.1.

Introduction.

The previous chapter of this thesis has shown that although infection with *T.annulata* alters the levels of MHC class II expression of MΘs, the actual levels of class II expressed by infected cells do not correlate with the T stimulatory ability of the infected cells. Activation of both memory and naive autologous T cells by infected cells has been shown to be dependent upon infected cell/T cell contact (Campbell *et al*, 1995) but the levels of T cell proliferation induced varies from one population of infected cells to another. This suggests that there is another factor or factors which are involved in the induction/maintenance of T cell proliferation. Cytokines are known to play an essential role in T cell activation and proliferation (Morgan *et al*, 1976; Kaye and Janeway, 1984). Therefore, the possibility that infected cells produce cytokines which are involved in T cell stimulation was investigated.

Normally monocytes and MΘs do not activate and induce the proliferation of $CD4^+$ T cells, unless they are presenting a specific antigen to T cells in the context of MHC class II molecules plus supplying a number of secondary messages to the T cells (*ie*, via accessory molecules and cytokines). This therefore means that cells infected with *T.annulata*, which possess the ability to activate and induce the antigen "non-specific" proliferation of autologous T cells do not act in the same manner as uninfected monocytes and MΘs. The mechanisms by which the infected cells bring about T cell activation/proliferation are not known. However, work described in this chapter highlights some of the mechanisms which could be responsible for the T cell responses induced by the presence of *T.annulata* infected cells.

There are two groups of molecules which are essential for T cell/accessory cell interactions - membrane bound costimulatory molecules and cytokines. Costimulatory molecules are expressed on the surface of accessory cells and interact with ligands on the surface of responding T cells during MHC class II/peptide/TCR interactions. One important example of these receptor ligand pairs are B7-1/B7-2 and CD28/CTLA-4, which are involved in T cell activation (Freeman *et al*, 1993; Hathcock *et al*, 1994). Contact between costimulatory molecules such as these initiates transduction of signals into the responding cell and results in molecular cascades which help initiate cellular activation.

Due to the difficulties in obtaining bovine specific identification of the infected cell/T cell contact requirements involved in *T.annulata* induced T cell activation and proliferation proved impossible. The possibility that other factors were also involved in these reactions were investigated. An area which I investigated was the production of cytokines by infected cells. Cytokines are chemical messengers produced by various groups of cells of the immune system, which have a wide range of effects on many cell types within and outwith the immune system. These molecules are generally released from the cell surface and diffuse into the extracellular milieu. As the molecules diffuse outwards they contact the surface of responder cells, binding to specific surface receptors, which transduce signals in to the responding cell.

The cytokines investigated included; Interleukin 1 (Dodds *et al*, 1994) (Mallardo *et al*, 1994), Interleukin 6 (IL-6) (Navarro *et al*, 1989), Interleukin 10 (IL-10) (de Waal *et al*, 1991) and Tumour necrosis factor alpha (TNFα) (reviewed by Beutler and Cerami, 1988; Armitage, 1994). The production of IL-1α, IL-6 and TNFα were specifically investigated as they are known to be involved in T cell activation and are produced by monocytes and MΘs. Also as noted in chapter I, during cases of Tropical Theileriosis symptoms are observed which are similar to those seen during sepsis (such as, anaemia and cachexia), which are known to be associated with the over production of these cytokines (Waage *et al*, 1989). Production of two major T cell stimulatory cytokines are IL-2 and IL-4 (Mauer *et al*, 1984; Smith, 1988; Paul, 1991) was also studied.

An important group of cells known to produce IFNγ are T cells of the T helper 1 (Th_1) subtype (Mossmann and Coffman, 1989). Infected cells have been shown to induce high levels of IFNγ, both *in vivo* (Nichani, 1995) and *in vitro* (Campbell, 1995). *In vitro* this cytokine has been shown to be produced by T cells stimulated with infected cells (Campbell *et al*, 1997), this is almost certainly also the case *in vivo*.

This cytokine is known to be an important stimuli for monocytes and MΘs, therefore the possibility that this molecule may be involved in the proliferation of infected cells was studied. After infection with *T.annulata*, populations of infected cells begin to rapidly increase in numbers both *in vivo* and *in vitro* (Barnett, 1977; Hulliger *et al*, 1964). IFNγ is a powerful stimulator of monocytes and macrophages and the presence of this cytokine may be involved in the rapid expansion of infected populations. IFNγ can act as a rescue signal for monocytes and MΘs, by inducing these cells to produce IL-1 and TNFα (Cotter *et al*, 1994; Mangan and Wahl, 1991). These two cytokines are able to inhibit the controlled death of these cells by apoptosis (Mangan and Wahl, 1991). IFNγ may therefore be an important message in the growth and development of *T.annulata* infected cells.

Assessment of the expression of secreted cytokine proteins or biological activities of any cytokines produced, would have been the most informative methods of determining possible roles for cytokines during *T.annulata* infection. However, at the time of study reliable assays for the detection of these bovine molecules were unavailable. Therefore, reverse transcriptase polymerase chain reaction (RT-PCR) was employed to investigate the production of cytokine mRNA. mRNA species specific for the cytokines in question were amplified from the parental and clonal cultures, including an unrelated line, infected with the related parasite *T. parva* (Naessens *et al*, 1985).

It was not possible to carry out fully quantitative PCRs for these cytokine transcripts, due to the lack of suitable internal PCR standards, which would be required for each PCR product. However, a semi-quantitative procedure was devised, which allowed comparisons to be drawn between the levels of cytokine mRNA expressed by different cell lines. This technique relies on identical PCR reactions being performed at varying

numbers of amplification cycles.

Each PCR reaction was set up six times and the PCR performed identically, apart from the number of amplification cycles performed. The samples were amplified at cycle numbers between 20 to 30 (rising in increments of 2 cycles). Comparison of the cycle number at which each PCR fragment becomes visible allows an estimation of the relative abundance of the cytokine mRNAs. This technique only allows mRNA expression levels to be compared between cell lines for the same primer pairs. The levels of IL-1α mRNA expressed by Ta.12929 can be compared only to the expression of IL-1α mRNA by the other cell lines but not to the expression of other mRNA species. This is due to differences in the priming efficiency between primer pairs and the technique relies on the assumption that primer pairs behave with identical efficiency in all PCR reactions. This method was used to investigate the expression of; IL-1α, IL-1β, IL-6, IL-10, TNFα and IFNγ mRNA by infected cells.

Following the establishment of the identity of cytokine mRNAs produced by these populations it was decided to investigate the relative levels of expression by *T.annulata* infected cell lines. This was to determine if the differences in T cell stimulatory ability (shown in chapter III) were due to variations in cytokine production by the various infected cultures. Following analysis of the cytokine mRNA species produced by these various infected cell populations, this technique was used in preliminary assays of the cytokine mRNAs produced by autologous T cells stimulated by either clone I or L. It was hoped that investigations into the production of cytokines by *T.annulata* infected cell stimulated T cells may elucidate possible mechanisms involved in parasite stimulated T cell proliferation and the immunopathology of Tropical Theileriosis.

Results.

Section 4.2.

Cytokine mRNA expression determined by RT-PCR.

During the investigation of the expression of cytokine mRNA by cells infected with *T.annulata* it was initially found that all the cell lines assayed possessed similar mRNA expression profiles when amplified with a 30 cycle PCR program. Figures 4.1 a and b show representative profiles produced by the parental line and clone I. All the *T.annulata* infected cells assayed expressed mRNA specific for; positive control, IL-1α, IL-1β and IL-6. IL-10 and TNFα mRNA were also found to be produced by cell line T.a 12929 and clones I and L. Clone G did not produce sufficient IL-10 or TNFα message for PCR product to be detected at 30 cycles (the highest level of amplification employed during these investigations).

The mRNAs specific for IL-2 and IL-4 were not detected from any *T.annulata* infected cell line assayed. However, Fig 4.1(a) shows that mRNA specific for IFNγ was detected from the parent line. This was the only *T.annulata* infected cell line assayed found to produce this cytokine mRNA (usually produced by T cells (Mossmann *et al*, 1989)). IFNγ mRNA was only detected on two out of five occasions (both soon after infection) suggesting that, although this infected cell line could produce IFNγ mRNA, it is not one of the cell line's major products. The production of cytokine mRNAs by the parental and clonal lines were assayed on five different occasions and the detection of the various mRNA species were constant.

A cell line infected with the related parasite *T.parva* was assayed for the same cytokines as those infected with *T.annulata* (Fig 4.1(c)). It was found that this cell

Fig. 4.1 **Agarose gels containing cytokine products detected following RT-PCR analysis of total RNA from infected cells of T.a 12929 and clone I.**

Photographs of agarose gels containing RT-PCR products obtained from *T.annulata* macroschizont infected cell lines, (a) T.a 12929, (b) clone I. Products present are specific for β-actin (positive control), IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, TNFα and IFNγ. Products specific for IL-2 and IL-4 were never found during analysis of any *T.annulata* infected cell line. Products were produced from 5μg of total RNA per RT reaction and 2μl of cDNA per PCR reaction after 30 amplification cycles. Size markers are produced by a Hae III restriction enzyme digest of λ-phage DNA, with fragment sizes given in base pairs (bp).

Fig. 4.1a

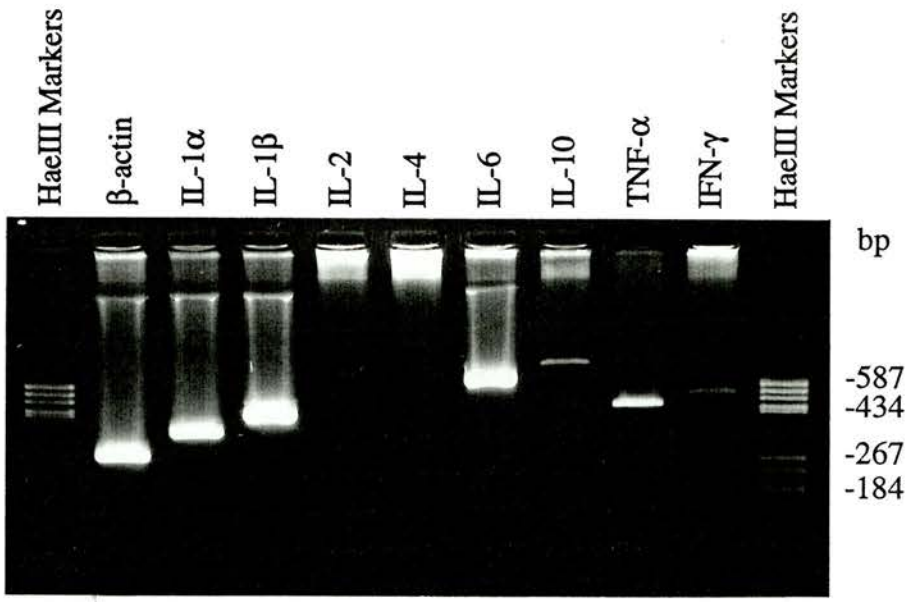
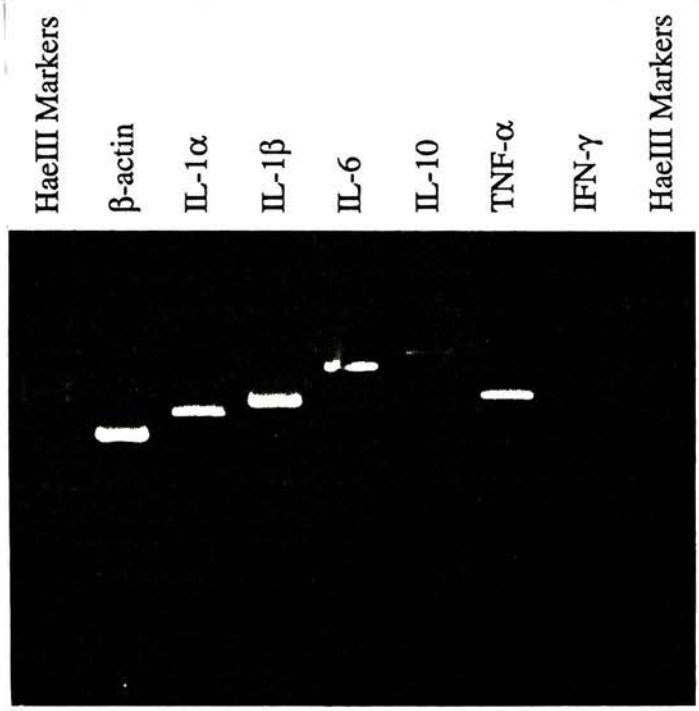


Fig. 4.1b



line, produced by infection of cells with *T.parva* sporozoites (Dr G. Russell, pers. comm), produced mRNA specific for not only the cytokines normally associated with production by T cells (IL-2, IL-4, IL-10 and IFNγ) but also mRNA specific for the T cell stimulatory cytokine IL-1α. However, the amount of IL-4 product produced was low, resulting in only a very faint band being visible in Fig. 4.1(c)

Samples of all the different PCR products were subjected to restriction digestion analysis and were shown to give the expected restriction patterns (Fig 4.2). These data show that when the RT-PCR products are digested with various restriction enzymes, the sizes of the digestion products match those which would be produced by digesting pure samples of the sequences in question, *i.e.* the PCR products contained only DNA sequences specific for the cytokines investigated.

Treatment of APCs with Mitomycin C is a standard practice during T cell proliferation assays (Fraser *et al*, 96) and irreversibly blocks DNA replication and stops the infected cells from overgrowing the T cells whilst in culture during proliferation assays. However, it was also necessary to determine whether treatment with mitomycin C inhibited cytokine mRNA production. Cytokine mRNA production by *T.annulata* infected cells after treatment with mitomycin C is shown in Fig. 4.3. Cells were harvested, washed and incubated in mitomycin C (see chapter II). Following this cells were washed twice and placed in culture for 7 days. These cells were then harvested and total RNA isolated as outlined in Chapter II, section 3. RT-PCR analysis showed that the infected cells were still producing cytokine mRNA specific for; IL-1α, IL-1β, IL-6, IL-10 and TNFα, 7 days after they had been incubated with mitomycin C.

Fig. 4.1c **RT-PCR analysis of a cell line infected with macroschizonts of the related parasite *T.parva*.**

Agarose gel showing RT-PCR products obtained from a cell line infected with the related parasite *T.parva*. Present are the products for, the positive control (β-actin), IL-1α, IL-2, IL-10 and IFNγ. These products were visible after 30 PCR amplification samples.

Fig. 4.1c

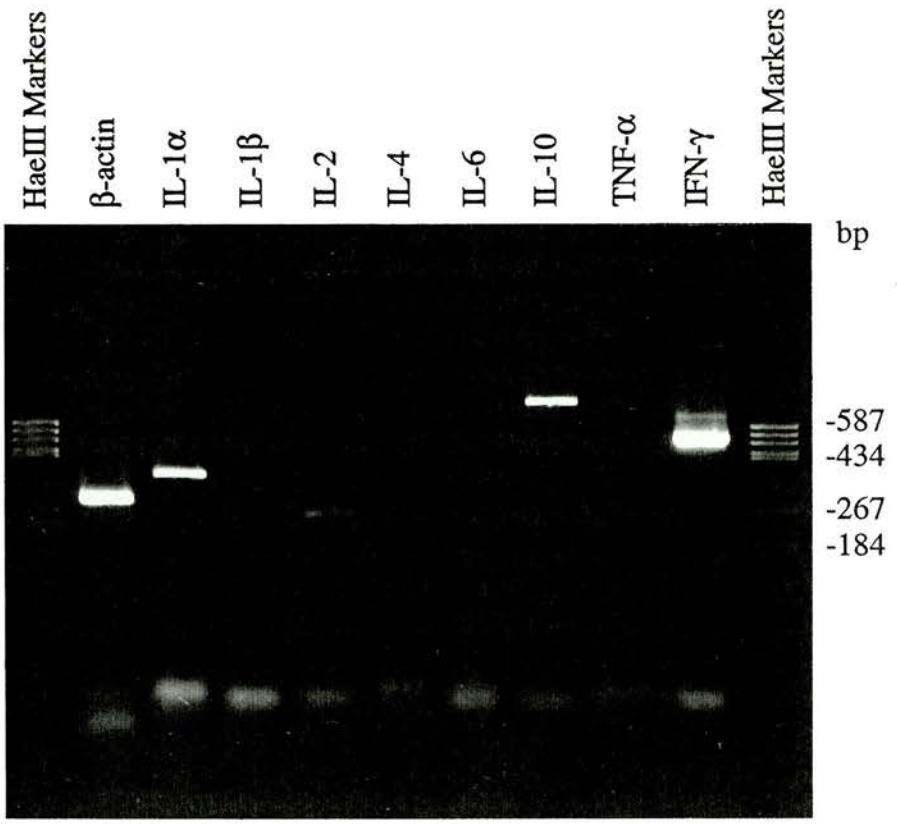

HaeIII Markers
β-actin
IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-10
TNF-α
IFN-γ
HaeIII Markers
bp
-587
-434
-267
-184

Fig. 4.2 **Restriction enzyme analysis of cytokine PCR product sequences.**

Restriction enzyme analysis of cytokine RT-PCR products. The enzymes used and fragments expected are as follows, IL-1α (Taq1) 161bp/204bp, IL-1β (PvuII) 169bp/263bp, IL-6 (BglII) 149bp/477bp, IL-10 (HaeIII) 306bp/439bp, TNFα (PvuII) 99bp/163bp/234bp, IFNγ (Pst1) 114bp/425bp. 15µl of each PCR product were digested for 2 hours at 37^0C with the appropriate restriction enzyme. The resulting fragments were visualised under U.V light on a 2% agarose gel stained with EtBr.

Fig. 4.2

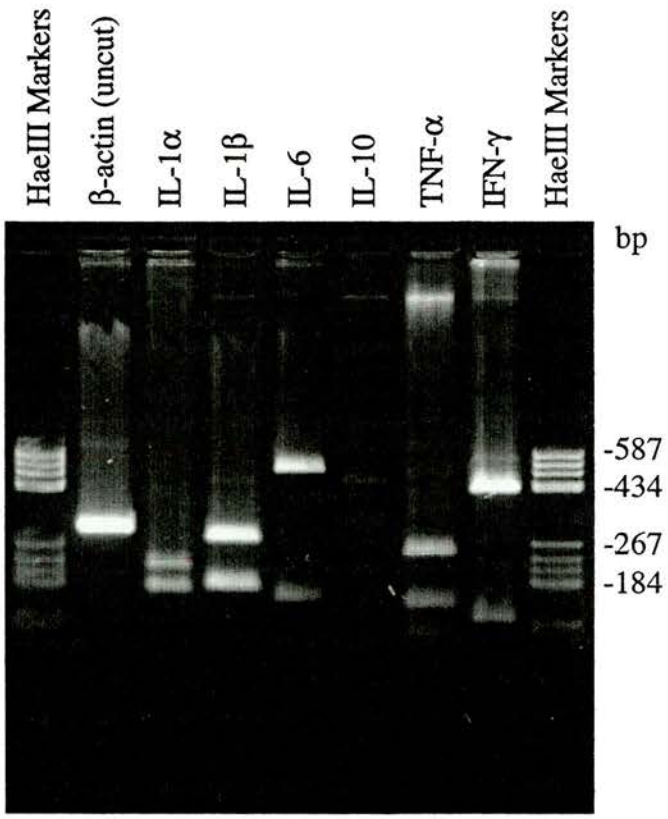
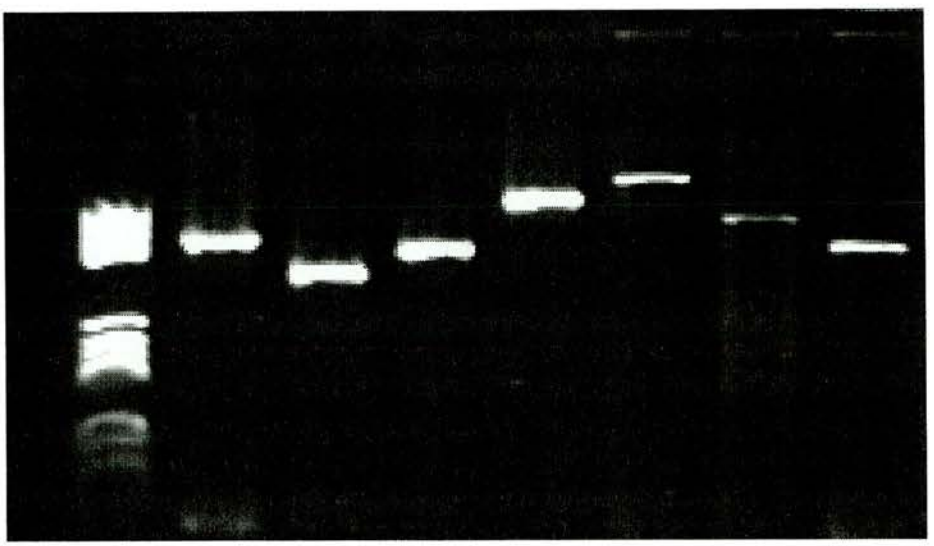

HaeIII Markers
β-actin (uncut)
IL-1α
IL-1β
IL-6
IL-10
TNF-α
IFN-γ
HaeIII Markers
bp
-587
-434
-267
-184

Fig. 4.3 **Analysis of the cytokine mRNA species expressed by macroschizont infected cells following treatment with the toxin mitomycin C.**

RT-PCR analysis of cytokine mRNA expressed by T.a 12929 on day 7 post mitomycin C treatment. The infected cells were incubated with mitomycin C at a concentration of 100μg/ml for 30 mins at 37^0C. The cells were then washed twice in PBS and placed in culture for 7 days, after which they were harvested and total RNA collected for RT-PCR analysis. Data shows that mRNA specific for, G3PD, IL-1α, IL-1β, IL-6, IL-10, TNFα and IFNγ continue to be produced 7 days after this treatment.

Fig. 4.3

Hae III G3PD IL-1α IL-1β IL-6 IL-10 TNFα IFNγ



Section 4.3.

Quantitation of cytokine mRNA expression by infected cells.

A number of PCR fragments could be detected from *T.annulata* infected cells after only 20-24 cycles (Fig 4.4 (a-d) and Table 4.1). The detection of products after these low levels of amplification means that the infected cell populations produce substantial amounts of these cytokine mRNA species. The samples were run on agarose gels, in groups of three, with increasing cycle number from left to right. β-actin was used as a positive control and it can be seen that all four infected populations produce β-actin mRNA detectable after only 20-22 cycles, with the intensity of these control bands increasing steadily as the number of amplification cycles increases.

Total RNA was collected from the infected cell populations on 2 separate occasions for use in limiting cycle analysis and the data produced showed that T.a 12929 and the clonal lines stabley produce the same levels of cytokine mRNA. Shown in Table 4.1 are the cycle numbers (rounds of amplification) needed to produce visible product from T.a 12929 and clone G, I and L. In general all of the cytokine mRNAs detected appear at the lower end of the amplification scale.

Limiting cycle PCR was used to investigate the relative amounts of each cytokine mRNA produced by each cell line (Fig 4.4 and Table 4.1). Fig. 4.4 a-d, show the levels of expression of mRNA specific for β-actin, IL-1α, IL-1β, IL-6, IL-10, TNFα and IFNγ by the parent line and clones G, I and L at different PCR cycle numbers (between 20 and 24 cycles for Ta. 12929/clones G & L and 60 to 30 cycles for clone I). Data concerning IFNγ production by clone I is not included in Fig. 4.4c, however, this PCR product was never detected during assay of any infected population except T.a 12929.

Fig. 4.4a **Semi quantitative RT-PCR analysis of cytokine mRNA expression by macroschizont infected cells of the T.a 12929 cell line.**

Data of limiting cycle RT-PCR analysis of T.a 12929 after 20-24 PCR amplification cycles showing products specific for β-actin, IL-1α, IL-1β, IL-6, IL-10, TNFα and IFNγ. Constant amounts of RNA (5μl) and cDNA (2μl) were used in all reactions.

Fig. 4.4a

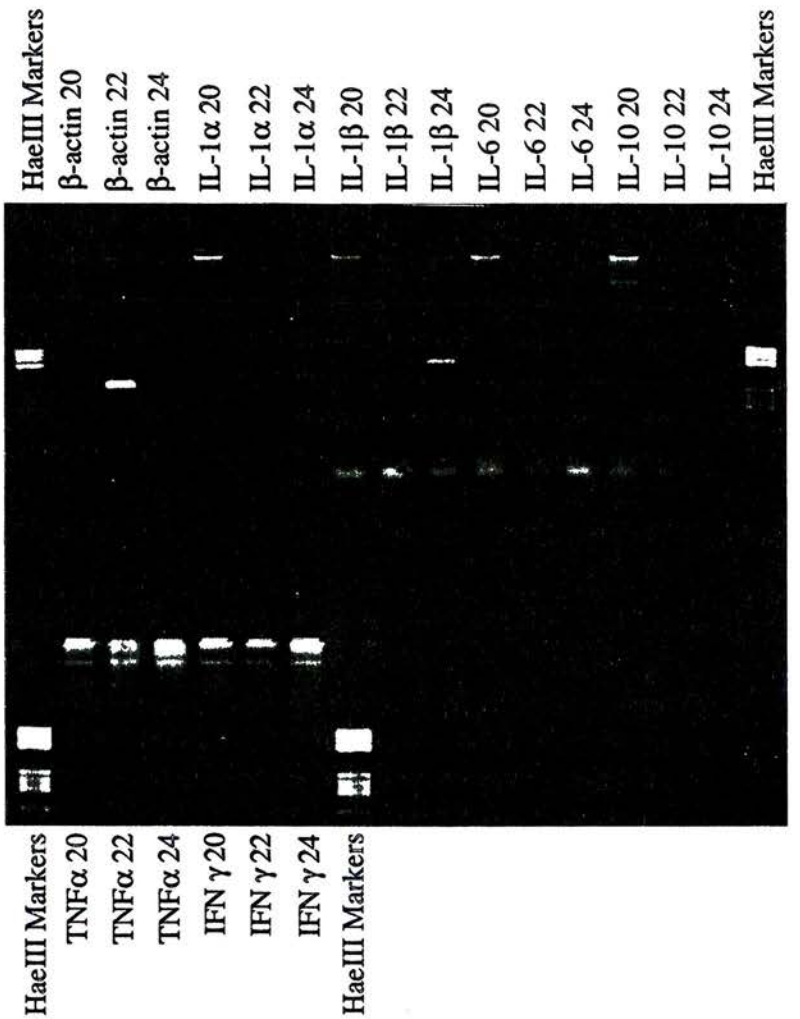

HaeIII Markers
β-actin 20
β-actin 22
β-actin 24
IL-1α 20
IL-1α 22
IL-1α 24
IL-1β 20
IL-1β 22
IL-1β 24
IL-6 20
IL-6 22
IL-6 24
IL-10 20
IL-10 22
IL-10 24
HaeIII Markers
HaeIII Markers
TNFα 20
TNFα 22
TNFα 24
IFN γ 20
IFN γ 22
IFN γ 24
HaeIII Markers

Fig 4.4b **Semi-quantitative RT-PCR analysis of cytokine mRNAs expressed by cells of clone G.**

Data of limiting cycle RT-PCR analysis of clone G after 20-24 PCR amplification cycles showing products specific for β-actin, IL-1α, IL-1β and IL-6.

Fig. 4.4b

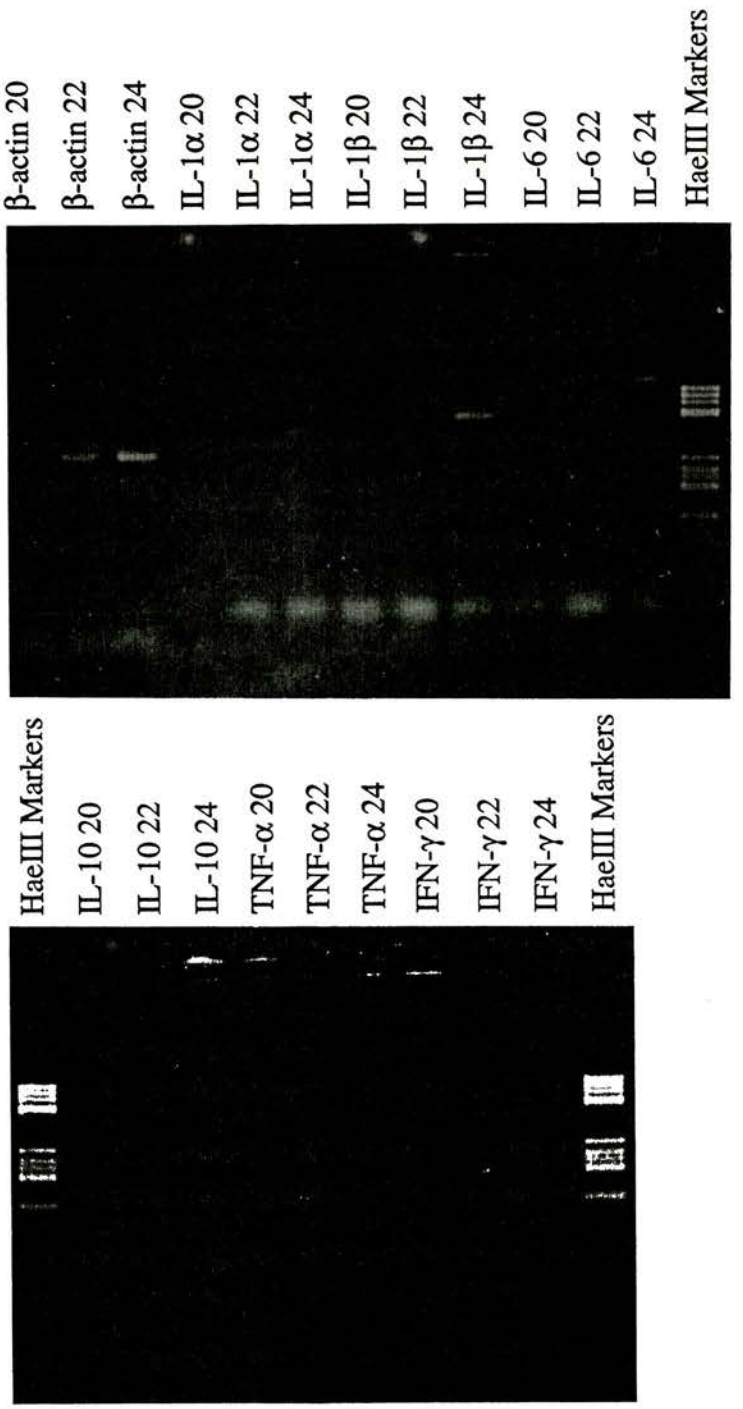

β-actin 20
β-actin 22
β-actin 24
IL-1α 20
IL-1α 22
IL-1α 24
IL-1β 20
IL-1β 22
IL-1β 24
IL-6 20
IL-6 22
IL-6 24
HaeIII Markers
HaeIII Markers
IL-10 20
IL-10 22
IL-10 24
TNF-α 20
TNF-α 22
TNF-α 24
IFN-γ 20
IFN-γ 22
IFN-γ 24
HaeIII Markers

Fig. 4.4c **Semi-quantitative RT-PCR analysis of cytokine mRNAs expressed by cells of clone I.**

Data of limiting cycle RT-PCR analysis of clone I after 26-30 PCR amplification cycles showing products specific for β-actin, IL-1α, IL-1β and IL-6.

Fig. 4.4c

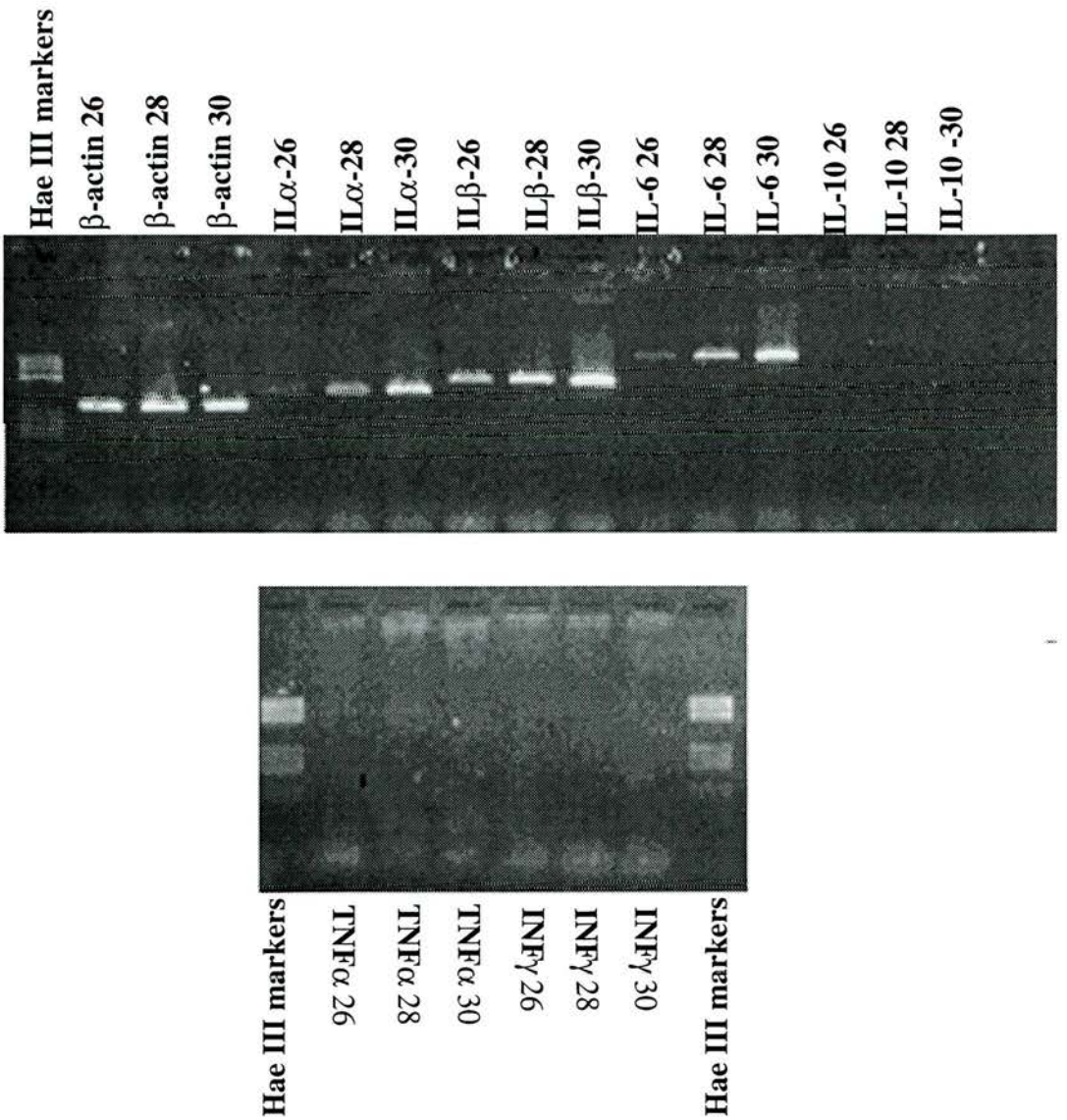

Hae III markers
β-actin 26
β-actin 28
β-actin 30
ILα-26
ILα-28
ILα-30
ILβ-26
ILβ-28
ILβ-30
IL-6 26
IL-6 28
IL-6 30
IL-10 26
IL-10 28
IL-10 -30
Hae III markers
TNFα 26
TNFα 28
TNFα 30
INFγ 26
INFγ 28
INFγ 30
Hae III markers

Fig. 4.4d **Semi-quantitative RT-PCR analysis of cytokine mRNAs expressed by cells of clone L.**

Data of limiting cycle RT-PCR analysis of clone L after 20-24 PCR amplification cycles showing products specific for β-actin, IL-1α, IL-1β, IL-6 and TNFα

Fig. 4.4d

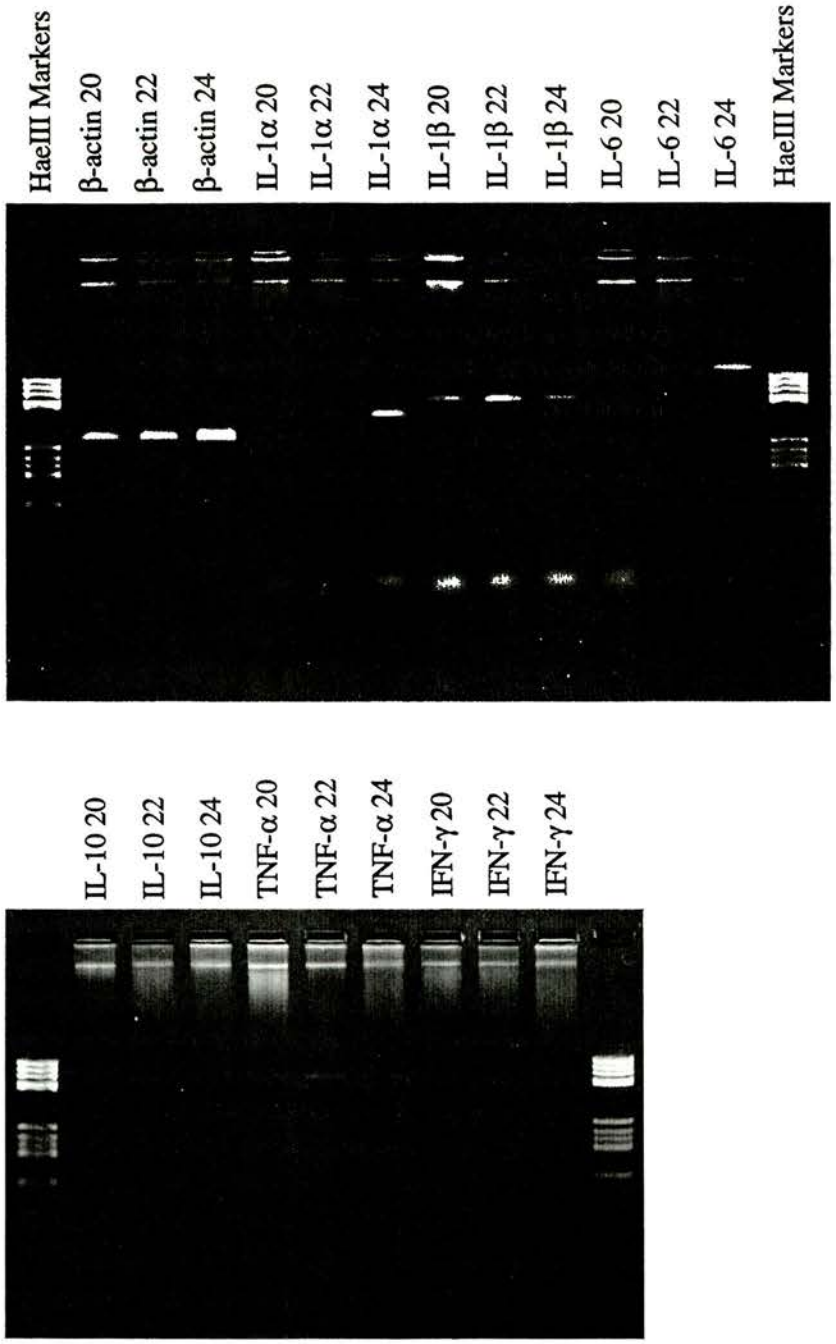

HaeIII Markers
β-actin 20
β-actin 22
β-actin 24
IL-1α 20
IL-1α 22
IL-1α 24
IL-1β 20
IL-1β 22
IL-1β 24
IL-6 20
IL-6 22
IL-6 24
HaeIII Markers
IL-10 20
IL-10 22
IL-10 24
TNF-α 20
TNF-α 22
TNF-α 24
IFN-γ 20
IFN-γ 22
IFN-γ 24

Table 4.1.

PCR product.	T.a 12929.	Clone G.	clone I.	clone L.
β-actin.	20	20	20	20
IL-1α.	24	24	26	20
IL-1β.	20	20	24	20
IL-6.	24	22	24	20
IL-10.	30	N.P.	28	24
TNFα.	22	N.P.	28	20
IFNγ	30	N.P.	N.P.	N.P.

Table 4.1 Cytokine mRNA expression by *T.annulata* infected cells.

Data produced from the semi-quantitative RT-PCR analysis of the macroschizont infected parent and clonal lines. RT-PCR analysis was carried out with constant amounts of mRNA (5μg per reaction) and cDNA (2μl per reaction) but the numbers of amplification cycles varied between 20 to 30 cycles (in steps of two). Products visible at low numbers of PCR cycles result from a mRNA message of higher abundance than those with are only visible after higher numbers of cycles. The table shows the cycle number at which each product became visible for each infected cell line. N.P.=PCR products not present.

Table 4.1 shows the cycle numbers at which the cytokine PCR products were detectable. PCR amplifies DNA templates exponentially, therefore, a product which becomes visible at 20 cycles results from a much more abundant source of mRNA than a product visible at higher numbers of cycles. Clone L produced the highest levels of mRNA for IL-1α, IL-1β, IL-6 and TNFα (visible at 20 cycles) and IL-10 mRNA visible at 24 cycles. The parent line and clones G and I express much lower levels of these mRNAs, with no detectable mRNA for IL-10 or TNFα being expressed by clone G. mRNA specific for IFNγ was detected from T.a 12929 on two out of five occasions, this product was only detected once during limiting cycle analysis and on that occasion the level of expression was only sufficient for a band to be visible after 30 cycles.

Section 4.4.

Quantitation of T cell cytokine expression by PBM stimulated with either clone I or clone L.

Table 4.2.

PCR product	Days. 1	2	3	4	5	6	7
G3PD.	20	20	20	20	20	20	20
IL-2.	24	24	24	22	26	26	26
IL4.	N.P.	26	26	24	26	30	N.P.
IFNγ.	22	20	20	20	20	26	20

Table 4.2 **T cell cytokine mRNA expression post incubation with clone I.** Semi-quantitative RT-PCR data produced by analysis of T cells stimulated with cells of clone I. RT-PCR was carried out using 5μl of mRNA and 2μl of cDNA per reaction. PCR amplification cycles varied in number from 20 to 30 (in steps of two). The table shows the number of amplification cycles necessary to produce visible product on an agarose gel stained with EtBr.

Table 4.3.

PCR product	Days. 1	2	3	4	5	6	7
G3PD.	20	20	20	20	20	20	20
IL-2.	24	22	24	22	26	26	26
IL4.	N.P.	26	26	26	26	N.P.	N.P.
IFNγ.	22	20	20	20	20	24	20

Table 4.3 T cell cytokine mRNA expression post incubation with clone L.

Semi-quantitative RT-PCR data produced by analysis of T cells stimulated with cells of clone L. RT-PCR was carried out using 5μl of mRNA and 2μl of cDNA per reaction. PCR amplification cycles varied in number from 20 to 30 (in steps of two). The table shows the number of amplification cycles necessary to produce visible product on an 2% agarose gel stained with EtBr.

Tables 4.2 & 4.3 contain data obtained after autologous PBM were incubated with infected cells of clone I or clone L. Seven cultures of autologous PBM and cells of either clone I or clone L were incubated for between 1 -7 days. Cultures of cells stimulated with either of these cell lines were harvested daily and total RNA isolated. Following the same limiting cycle PCR protocol as previously described, the abundance of T cell derived cytokine mRNAs were investigated. The data presented above shows clone L stimulated T cells produce slightly higher levels of IL-2 and IFNγ and slightly lower levels of IL-4 than clone I stimulated T cells. However, the

differences are transient and generally very little difference in the levels of T cell stimulatory, T cell derived cytokine mRNAs are detected between cells stimulated with either clone I or L.

Section 4.5. **Discussion.**

The investigations detailed in the previous chapter show that the levels of MHC class II expressed by infected cells do not correlate with the T cell stimulatory ability of *T.annulata* infected cells. Once this had been determined, other stimuli which may be involved in the "non-specific" T cell activation and proliferation were investigated. The data presented in this chapter show that macroschizont infected cells constitutively produce a wide range of cytokine mRNAs.

It is perhaps not surprising that Ta. 12929 and the clones derived from it express cytokine mRNAs associated with monocytes and MΘs, since the original infected cell line was produced from $CD14^+$ blood monocytes. However, the finding that these cytokine mRNAs were constitutively produced at stable levels was unexpected, as cytokine expression is usually under tight physiological control, with these molecules only being expressed after recent stimulation. Also it was not expected that these lines would be found to express so many cytokine mRNAs at the same time.

T cell activation.

The discovery that *T.annulata* infected cells produced a number of cytokine mRNAs suggested possible mechanisms for induction of the T cell activation observed during *T.annulata* infection (Glass and Spooner, 1990; Campbell *et al*, 1995). As previously mentioned, studies showed the activation of T cells to be reliant upon autologous T cell/infected cell contact (Campbell, 1995). This work involved the culture of freshly isolated, autologous T cells with infected cells in the presence or absence of a semi-permeable membrane. It was found that when T cells were allowed to contact infected cells, they quickly became activated and within 2-3 days expressed predominantly a Th_1 (Mosmann and Coffman, 1989) phenotype (Campbell, 1995). This was in contrast

to the culture of T cells separated from infected cells by a semi-permeable membrane, where T cell activation/proliferation was not induced. The membrane allows free diffusion of any soluble factors produced by the infected cells (such as cytokines) but not contact of infected cells with the surface of the T cells.

Macroschizont infected cell derived cytokines & T cell activation.

As shown in this chapter the infected cells produce a wide range of cytokine mRNAs and of these the majority are known to have affects upon T cells. Infected cells have been shown to produce mRNAs specific for; IL-1α, IL-1β, IL-6 and IL-10.

IL-1 has long been known to possess T cell stimulatory capabilities (Kaye *et al*, 1984), with the ability to stimulate IL-2 production by T cells and upregulating the expression of the IL-2 receptor (IL-2R). The exact roles of the two related IL-1 molecules (IL-1α and IL-β) are still under investigation. However, it has been suggested that IL-1α binds more efficiently with the type I receptor (Dinarello *et al*, 1991), whilst IL-1β binds better to the type II receptor (Dower *et al*, 1992). This data along with that of Sims *et al* (1993) suggests that the majority of the biological activities of IL-1 are due to IL-1α/Type I receptor interactions. This is possibly due to the fact that the cytoplasmic domain of the type I receptor is 215 amino acids (aa) long (Sims *et al*, 1993), whilst that of the type II receptor is only 29 aa, suggesting that this receptor is unable to transduce messages in to the cell. The T cell stimulatory ability of IL-6 was discovered by two groups simultaneously (Lotz *et al*, 1988) (Uyttenhove *et al*, 1988). Later studies have shown that IL-1 and IL-6 synergise during T cell activation, IL-1 stimulating IL-2 production and IL-6 enhancing IL-2 responsiveness (Van Snick, 1990).

IL-10 is a cytokine which possesses regulatory abilities over a number of cellular activities and like IL-6 can be produced either by monocytes/MΘs or T cells (Fiorentino *et al*, 1989; Mosmann and Moore, 1991). This cytokine has been shown to down-regulate cytokine production by activated monocytes (de Waal *et al*, 1991) and to inhibit the proliferation of all three groups of bovine T cells (Brown *et al*, 1994). It would appear that the main affects of IL-10 are upon the APCs involved in

T cell stimulation, as this cytokine has not only been shown to down-regulate cytokine production but also to directly effect the antigen presenting capacity of monocytes and MΘs (Fiorentino *et al*, 1991). Evidence also exists for the down-regulation of MHC class II expression on MΘs (de Waal *et al*, 1991) (Howard *et al*, 1992) and it has also been suggested that the presence of IL-10 is responsible for the inhibition of production or function of a MΘ-membrane bound costimulatory molecule involved in the activation of T and NK cells (Moore *et al*, 1993). Workers have also shown IL-10 to be involved in the inhibition of surface expression of ICAM-1 (Leeuwenberg *et al*, 1994) and B7 molecules (Willems *et al*, 1994) and to down-regulate the synthesis of the superoxide anion (Niro *et al*, 1992). IL-10 is therefore widely implicated in the down-regulation of APC function and T cell activation.

During this investigation the production of IL-12 by infected cells was briefly studied and some evidence was found for the production of this cytokine by infected cells. However, it was later discovered that the primers used were specific for one of the two IL-12 chains (Trinchieri, 95) the p35 chain of IL-12. Recent data suggests that this part of the molecule is constitutively expressed by monocytes and MΘs and the data collected is therefore inconlusive as to whether this cytokine is actually expressed. But in recent months whilst working with Dr J. Campbell at the Center for Tropical Veterinary Medicine (Edinburgh University), we have found that infected cells also express mRNA specific for the p40 subunit of IL-12. This shows that infected cells are producing both mRNAs necessary for the production of functional IL-12 and suggests that the cells are making this cytokine.

IL-12 is a potent activator of both T cells and NK cells and induces these cells to produce IFNγ which activates MΘs (Ijzermans and Marquet, 1989; Bancroft *et al*, 1994). For MΘs to be producing message specific for both IL-10 and IL-12 at the same time is unusual and suggests that the immunoregulatory mechanisms are not functioning as normal (Reed, 1995). Reed suggests that the presence of antagonistic cytokines such as IL-10 and IFNγ at the same time during *T.cruzi* infections is an important factor in determining succeptibility.

The production of different cytokines within an immunological reaction allows the

regulation of cellular immune responses. Following the initial activation of a cell by cell/cell contact and cytokine stimulation, the production of cytokines can be used to regulate the progressing immune response with great subtlety. Cytokines do not simply produce reactions by their own actions, they are known to synergise with others, to augment or decrease cellular activation and responses. An example of this is the synergy showed between IL-1 and IL-6 during T cell activation (Houssiau *et al*, 1988). Cytokines can also act antagonistically, one example of this can be seen between the reactions induced by IL-10 and IL-12 during responses to *L.donovani* (Ghalib *et al* 1995). Here production of IL-10 has been shown to exacerbate the infection (by down regulating Th_1 responses), whilst the induction of IL-12 production has been shown to aid in clearance of this protozoan parasite (by inducing production of IFNγ which activates macrophages, leading to the removal of parasite).

The constitutive production of such a large number of cytokine mRNAs by *T.annulata* infected cells suggests that immune responses induced by infected cell/autologous T cell contact are not under the normal constraints observed during T cell activation. The majority of the cytokine mRNAs found to be produced by *T.annulata* infected cells are specific for T cell stimulatory cytokines (IL-1α, IL-1β and IL-6). When data of cytokine mRNA production by the parent line and clones is viewed in relation to data collected following stimulation of T cells by these infected cells, it can be seen that the infected cells producing higher levels of mRNA (of cytokines known to stimulate T cells), induce higher levels of T cell proliferation.

Clone L produces cytokine mRNAs specific for IL-1α, IL-1β and IL-6 at considerably higher levels than clone I, with clone L cytokine mRNAs detectable approximately four to six PCR cycles before those produced by clone I. These results correlate with the T cell proliferation induced by these two clones (as shown in Chapter III). Clone G and the parental line produce these three T cell stimulatory cytokine mRNAs at levels detectable between those of clone I and clone L and also show induction of T cell proliferation above that of clone I but below that of clone L. These results would suggest that the amounts of mRNAs specific for IL-1α, IL-1β and IL-6 are closely linked to the levels of T cell proliferation induced by the different populations of

infected cells. As noted we know that cell contact is essential for *T.annulata* induced T cell proliferation but this is the first evidence to suggest that parasite infected cells are able to produce cytokines which play an essential role in controlling non-specific T cell proliferation, after infected cell/T cell contact. However, the exact role of each cytokine and also the levels of translation of each mRNA species into product must be investigated further, to allow us to delineate the reactions which are essential for the control of T cell proliferation by infected cells.

IL-10 production.

The expression of IL-10 mRNA by all of these cells is generally low, with RT-PCR product only being detected at high cycle numbers for the parental line (30 cycles), clone I (28 cycles) and with no IL-10 product being detected by clone G. Paradoxically clone L was shown to produce levels of IL-10 mRNA detectable at 24 cycles, with this result apparently contradicting T cell proliferation data produced when clone L was used to stimulate T cells. IL-10 is a cytokine which is capable of down-regulating the T cell stimulatory abilities of APCs (Leeuwenberg *et al*, 1994; Willems *et al*, 1994). One would not expect to find cells expressing high levels of T cell stimulatory and down-regulatory cytokines at the same time.

There are a number of possible reasons why this apparently high level of IL-10 mRNA expression may not effect the T cell proliferation induced by clone L. One is that the levels of T cell stimulatory cytokines produced are such that they override the effect of the IL-10 produced. Another is that IL-10 is only expressed as mRNA by the cell lines and that these molecules are not translated into functional IL-10. It is also possible that the presence of the parasite within the infected cells overrides the effect of IL-10 and that costimulatory ability of infected cells is not altered by the presence of this cytokine. The production of IL-10 mRNA by infected cells does not seem to lower the expression of class II or other cytokine mRNAs by infected cells. Unfortunately this hypothesis could not be tested further by analysis of accessory molecule expression.. The role of IL-10 produced by parasitised cells may be to down regulate activities of other uninfected cells, again in an attempt to subvert the immune

response.

From other work on the immune responses observed during *T.annulata* infections and *in vitro* T cell proliferation/RT-PCR cytokine assays it is known that the T cell response which predominates is that of a Th_1 type response (also that NK cells are probably activated) (Nichani, 1994; Campbell, 1995; Campbell *et al*, 1997). These data suggest that the cytokines which aid in determining the immune response in the presence of *T.annulata* infected cells are those which induce and maintain Th_1 type responses. Therefore it would appear that, the presence of Th_1 T cell stimulatory cytokines such as IL-1and possibly IL-12 override those of other cytokines such as IL-10.

T cell stimulation and IL-1α production.

A T cell line infected with the related parasite *T.parva* produced a different range of cytokine mRNAs, including IL-1α, IL-2, IL-4, IL-10 and IFNγ. The production of IL-1α by the *T.parva* infected cell line is surprising as T cells do not normally produce this cytokine (Mossmann and Coffman, 1989). Cells infected with either of these parasites induce contact dependent proliferation in naive autologous T cells (Campbell *et al*, 1995; Goddeeris and Morrison, 1987), suggesting that these parasites share a common mechanism for the stimulation of T cells. The contact dependency of T cell stimulation and the presence of IL-1α (which can be expressed in a membrane bound form (Kurt-Jones *et al*, 1985)) mRNA in both types of infected cells suggests that this cytokine may be of great importance in the induction of T cell proliferation by infected cells.

The induction of T cell proliferation depended partly upon the state of growth of the infected cells. If the infected cell lines or clones were not passaged approximately 18 hours before the non-specific proliferation assays were initiated, the levels of T cell proliferation observed were considerably reduced. This suggests that the ability of the infected cells to induce T cell proliferation may relate to the condition of the infected cells, as passaging the culture 18 hours earlier stimulates growth of infected cells. As was discussed in chapter III, cultures which are not passaged before use lose

condition, as suggested by the accumulation of detritus within the culture and alterations in the expression of surface molecules. This loss of condition may also effect the expression of cytokines by the infected cells.

Due to the knowledge that the state of growth of the infected cells altered their ability to induce T cell proliferation, RT-PCR analysis of cytokine mRNAs was only performed on cultures supplied with fresh medium 14 to 18 hours before analysis. Further studies are required to investigate whether the production of cytokine mRNAs decrease as the length of time after passage increases.

Previous studies have shown that non-specific activation of T cells also occurs *in vivo* (Campbell *et al*, 1995). During *T.annulata* infection, T cell blasts are found surrounding foci of macroschizont infected cells in the medullary region of lymph nodes draining the site of infection. These cells are unlikely to be stimulated by classical antigen presentation mechanisms, as they appear in the wrong anatomical location and much sooner than one would observe normally (Campbell *et al*, 1995; Campbell, 1995) and are possibly stimulated by a superantigen like activity of infected cells. Data shown in this thesis suggests that an important mechanism by which *T.annulata* induces T cell activation *in vivo* is through the production of T cell stimulatory cytokines by the infected cells. I have also shown that it is possible for infected cells to produce mRNA specific for the cytokine IFNγ, although this would appear to be a minor product which is not produced by the majority of infected cells. The identification of this cytokine mRNA within a culture derived from monocytes and MΘs is unusual. This suggests that the presence of the parasite has far ranging consequences for cytokine production.

The two occasions when IFNγ was detected within mRNA isolated from the parent line were both soon after infection of the $CD14^+$ cells with sporozoites. At this stage it will be important for the parasite to successfully "transform" the cell, allowing survival/proliferation. It may be that induction of IFNγ production by infected cells at these early stages are part of the mechanisms which allow the survival of the parasitised cell and ensure the production of more macroschizonts (Campbell *et al*, 1997).

For the T cell stimulatory ability of these different populations of infected cells to be assessed in full and the importance of cytokine production by infected cells to be gauged, actual levels of biologically active cytokines must be assessed. The roles of individual or mixtures of cytokines within *T. annulata* induced T cell stimulation could be investigated by the use of cytokine specific mAbs during blocking studies. However, the data contained within this thesis suggest that cytokines produced by infected cells are able to potentiate T cell stimulation induced by contact with infected cells.

Assessment of T cell stimulatory cytokine production by T cells stimulated with macroschizont infected cells.

To assess the possibility that proliferation of macroschizont infected cell stimulated, autologous T cells involves an autocrine cytokine loop, the production of T cell stimulatory cytokines by autologous T cells stimulated with the different *T.annulata* infected lines/clones were assessed by limiting cycle RT-PCR analysis. These experiments formed a preliminary investigation, conducted to assess T cell cytokine involvement in macroschizont stimulated T cell proliferation.

The data collected from this experiment suggests that, although the infected cells stimulating autologous T cells express very different levels of T cell stimulatory cytokine mRNA and induce very different levels of T cell proliferation, they did not induce responding T cells to produce different levels of T cell stimulatory cytokine mRNA. One might hypothesise that T cells stimulated with clone L would produce IL-2 at markedly higher levels, with IL-2 products being detected at lower cycle numbers than from T cells stimulated with clone I, however, this was not the case. Obviously much more detailed investigations into the mechanisms of *T.annulata* stimulated T cell proliferation are needed but these data do suggest that it is the infected cells which form the primary stimuli for macroschizont infected cell induced T cell proliferation and that production of T cell derived cytokines, which have been shown to enhance T cell proliferation are not responsible for the differences in T cell proliferation induced by the clonal lines.

Inflammatory cytokine production and T.annulata induced pathology.

Cytokines such as IL-1, IL-6 and TNFα have long been associated with the initiation of inflammatory reactions and the induction and exacerbation of sepsis (Waage *et al*, 1989). Many of the clinical characteristics associated with Tropical Theileriosis (reviewed by Neitz, 1957; Barnett, 1977; Samantery *et al*, 1980) are also observed in the presence of high levels of inflammatory cytokines. These include fever, anaemia, oedema, dypsnoea, loss of appetite, lethargy and in severe or protracted cases, bowel necrosis.

Cells infected with *T.annulata* produce high levels of inflammatory cytokine mRNAs, especially TNFα. This cytokine is a potent inducer of fever and has also been linked to the production of anaemia, muscle wasting and necrosis (Sileghem *et al*, 1994; Bielefeldt-Ohmann *et al*, 1989). These symptoms are observed in cases of acute Tropical Theileriosis. Although the anaemia and fever observed have been linked to the formation of piroplasms in RBCs (Uilenberg *et al*, 1993), followed by the lysis of piroplasm infected cells, there are documented cases where infections have proved fatal before the detection of piroplasms (Pipano *et al*, 1971). Interestigly the cell line infected with *T.parva* does not produce mRNA specific for TNFα, and *T.parva* infections are not characterised by high levels of anaemia (Wilde *et al*, 1967).

The role of cytokines in other parasitic infections.

There are a number of other intracellular parasites which are able to parasitise monocytes and MΘs, two examples being *Leishmania sp* and *Toxoplasma gondii*. The cytokine profiles of cells infected by these parasites have also been implicated in parasite growth and development. It has been suggested that IL-6 is involved in the intracellular replication of *T.gondii* (Beaman *et al*, 1994). Work showed that the presence of IL-6 could enhance *T.gondii* replication within murine peritoneal MΘs but that addition of IFNγ to the culture medium resulted in killing of the parasites. Also it was noted that addition of TNFα to the IFNγ treatment enhanced parasite killing. This is presumably due to the activation of the MΘs by the IFNγ or IFNγ/TNFα, leading to intracellular reactions which destroy the parasite. Also in cases of

L.donovani (Ghalib *et al*, 1995) and *L.major* (Wang *et al*, 1994) the induction of MΘ activation in the presence of IFNγ, results in the destruction of the parasite within infected cells.

The presence of these cytokines do not appear to have the same effects on *T.annulata* infected cells as they do on *Toxoplasma* or *Leishmania*. My work has shown that the infected cells themselves express numerous mRNAs specific for T cell activating cytokines. It is also known that once T cells are activated by infected cells they begin to express a number of cytokines, including IFNγ (Campbell *et al*, 1995; Campbell, 1995). Experiments performed by Nichani (1994) showed that large amounts of biologically active IFNγ were present in efferent lymph samples taken from animals infected with *T.annulata*. One can therefore assume that the presence of elevated levels of IFNγ do not have the same affect upon *T.annulata* infected MΘs as they do for *Leishmania* or *Toxoplasma* infected MΘs. This suggests that the mechanisms by which *T.annulata* controls infected cell function differ from those of these other intracellular parasites. They are able to evade destruction even though there are probably high levels of MΘ activating factors such as IFNγ, IL-1, IL-6 and TNFα, within the intercellular milieu.

One very important result of this work has been the discovery of a correlation between the activation of naive autologous T cells and the levels of IL-1α produced by the infected cells used as a T cell stimuli. This however, remains a theory and further work is required to prove a direct link between T cell activation/proliferation and infected cell IL-1α production. To do this one must possess anti IL-1α Abs, which are capable of blocking IL-1α/IL-1αR interactions. If incubation of infected cell lines which produce high levels of this cytokine, in the presence of anti IL-1α Abs results in the abrogation of the high levels of T cell proliferation (as those observed post incubation of T cells with clone L), one may conclude that IL-1α is one of the main T cell stimulatory signals involved in *T.annulata* T cell activation/proliferation. These methods could also be applied to study the roles played in T cell proliferation and pathogenesis by other cytokines.

Chapter V.

In vivo assessment of the pathology and protection produced following immunisation of cattle with clone I or clone L.

Section 5.1.

Introduction.

A molecular vaccine against *T.annulata* has proved difficult to produce. The only suitable vaccines currently available rely upon attenuated macroschizont infected cells. Vaccination with live attenuated infected cell lines produces solid immunity against *T.annulata* and provides an extremely reliable method of protecting cattle against this parasite. Vaccine regimes vary but generally consist of subcutaneously immunising animals with 1-2x10^6 macroschizont infected cells (Pipano, 1971; Ouhelli *et al*, 1989). Once attenuated cell lines generally provoke few clinical signs but do provide the immunised animals with solid immunity against sporozoite challenge.

Cell lines are attenuated over 2 to 3 years of *in vitro* culture. The mechanisms involved in attenuation are not well understood - there is evidence for alteration of parasite gene expression, and the apparently random selection of clonal parasite populations over time (Sutherland *et al*, 1996).The necessity of long periods of culture involves much work and incurs high costs and results in poorly defined vaccine cell line populations. Therefore, methods which could speed the attenuation/selection process of putative vaccine lines would prove a boon to vaccine producers and users alike.

Recent new work has lead to an increase in the understanding of factors underlying the production of pathology of tropical theileriosis. Particularly - the induction of non-specific T cell activation by *T.annulata* infected cells *in vivo* critically disrupts the formation of normal immune responses (Campbell *et al*, 1995; 1997). Production of very high levels of IFNg by these activated T cells may actively aid parasite growth (Campbell, 1995). Also many of the signs associated with *T.annulata* infection such as fever, cachexia and anemia may be attributable to the actions of proinflammatory

cytokines such as IL-1α and TNFα (Waage *et al*, 1989; Bielefeldt-Ohmann *et al*, 1989).

Aberrant T cell activation and the presence of high levels of cytokines may therefore play important roles in the pathogenesis of tropical theileriosis. It was hypothesised that a cell line which induced low levels of T cell proliferation and expressed low levels of proinflammatory cytokines had the potential to be used as a vaccine line without inducing pathogenic side effects. In this thesis cell lines were produced with stabely produced different amounts of T cell proliferation and proinflammatory cytokine mRNA species. Two of the clonal cultures previously characterised were chosen for use in a small scale clinical trial. Of the clones available, clone I was selected due to it's low T cell stimulatory ability/cytokine mRNA production and clone L for it's high T cell stimulatory ability and cytokine mRNA production. This was to compare the pathogenesis induced by a cell line inducing high levels of T cell proliferation and expressing high levels of proinflammatory cytokine mRNAs, with a low T cell activation/low cytokine mRNA producing cell line.

Due to the high costs incurred when working with cattle (*ie*, the cost of animals, housing and feed) this initial trial consisted of two groups of three Hereford/Friesian 3 year old heifers. All animals were in good health prior to the trial and were housed at Dryden Farm, Roslin. Each animal in each of the two groups of three, received a subcutaneous inoculation of $1x10^6$ infected cells of either clone I or clone L into the prescapular region. Following this animals were monitored for 24 days. Parameters measured included, temperature, packed cell volumes (PCV), total erythrocyte count (TEC), total leukocyte count (TLC), mean erythrocyte volume (mRBCv). The appearance of macroschizont infected cells and piroplasms in the periphery were also monitored and blood samples taken in an attempt to reisolate parasite material from the recipient hosts. Lymph node biopsies would probably have proved a better method of detecting infected cells. But this was not possible due to the large size of the animals (approximately 750kg) and the lack of a suitable crush, which would have allowed samples to be taken safely. After this initial period of study the animals were rested for 1 month and then with the addition of two unimmunised control animals challenged with a potentially lethal dose of *T.annulata* sporozoites.

Results.

Section 5.2.

Immunisation trial.

The clinical signs of all six animals used in the initial immunisation trial were monitored after administration of either clone I or L. Representative data from animals within the two groups (No.s 13274 and 13271) are displayed in Fig. 5.1 (a-e), with parameters shown including: temperature, PCV, TEC, TLC, and mRBCv. Data from the remaining four animals is shown in Appendix section 5 (figures contained in the appendix are designated App. followed by an identifying number, *i.e.* App 5.1.).

Included in each graph are the ranges of normal data for animals of similar age, sex and breed (taken from Veterinary Haematology, Schalm, Jain & Carroll). Statistical analysis was performed upon data collected from the six animals and consisted of 1 or 2 sided t-test analysis of data from days 1-10 (2 sided), 11-17 (1 sided) and 18-24 (1 sided), when comparing responses to clone I against those induced by clone L. These time periods were chosen following analysis of data obtained post immunisation of with cell line vaccines (Nichani, 1994). The data collected showed that the progress of reactions post immunisation could be broken down in to these three periods.

One sided t-tests were implemented when the probable outcome of the experiment had been predicted prior to immunisation and two sided tests used if the result of the experiment had not been predicted before immunisation. Statistical analysis of the period day 1 to 10 inclusive for all these parameters showed that there were no significant differences between the responses of animals immunised with clone I and those immunised with clone L. No animals became seriously ill after immunisation with either clone I or L and all infections were self clearing. Checks of all animals temperatures and blood counts were subsequently made on days 27, 31, 36, 38, with parameters approaching those prior to immunisation by days 36/38 and all animals proved resistant to an approximate LD50 dose of Gharb sporozoites.

Table 5.1

Parameter.	*days 1-10.*	*days 11-17.*	*days 18-24.*
Temperature.	N.S	**P<0.05**	N.S
PCV.	N.S	**P≤0.01**	**P<0.05**
TEC.	N.S	N.S	N.S
TLC.	N.S	**P<0.05**	N.S
RBC vol.	N.S	N.S	N.S

Table 5.1 Statistical analysis of data collected after immunisation.

Student t test analysis of differences between data collected following immunisation of cattle with either clone I or clone L. No significant differences were found during the first phase of the trial (days 1 to 10). During the second and final phase of reaction animals to the clones differences were found between groups I and L. These included differences in temperature, PCV and leucocyte counts.

Temperatures.

For the first 13 days of the trial five animals did not exhibit fever (Fig. 5.1a, App 5.1/5.2). Animal 13249 (group L) showed a temporary increase in temperature on day 7 (39.75^{0}C), followed by a return to normal temperatures until day 12. However, between days 13 and 14 the temperatures of animals immunised with clone L began to rise and stayed above the febrile response level (39.5^{0}C) until day 15/16, peaking at between 40.4^{0}C to 40.9^{0}C on day 14. The temperatures of animals contained in group I did not increase as much as those of group L, although animals 13274 and 13254 did exhibit two days of mild fever (39.55^{0}C and 39.65^{0}C respectively; Fig. 5.1a and App 5.1.).

PCV analysis.

Analysis of the PCVs (Fig. 5.1b, App 5.3/5.4) after immunisation showed that the packed cell volumes of both groups began to decrease within 2-4 days after administration of the infected cells. After immunisation with either clone I or L, PCVs of all animals decreased steadily until day 14 but one can see both from Fig. 5.1b and Table 1, that the PCV levels of animals immunised with clone I are significantly higher than those immunised with clone L between days 13 to 16. Also only animals immunised with clone L exhibited levels which fell to the lower end of the normal range of PCVs for 3 year old heifers (day 14).

Total erythrocyte counts and mean RBC volumes.

Investigation of the total erythrocyte counts (Fig. 5.1c, App 5.5/5.6) of animals from group I and L showed that there was little difference between the two groups' reactions to the immunising cell lines. All six animals exhibit an initial rise in RBC number, after which the TEC numbers in both groups fluctuate widely over the following twenty days. The TECs of animals immunised with clone L are generally lower than those immunised with clone I but there were no significant differences between the two groups. The mean RBC volumes (Fig. 5.1d, App 5.7/5.8) did not differ greatly between the two groups. Both groups of animals show a rapid initial decrease in RBC volumes between day 0-4/5, after which cell sizes continue to fluctuate for the remaining twenty days. However, an interesting point to note is the apparently synchronous fluctuations in mRBC vol in animals of group L between days 0 to 10.

Total leukocyte counts.

The total leukocyte numbers of animals in both groups vary greatly over the first 13 days but there were no significant differences ($P>0.05$) in the levels expressed by the two groups of animals (Fig. 5.1e, App 5.9/5.1.0). However, statistically different differences do appear between the two groups during days 14 - 16 ($P<0.05$). Between these days the leucocyte levels of animals in group L fall faster than those in group I and reach levels ($3.0x10^3$-$5.1x10^3$ cells/mm^3) well below the minimum normal range of bovine leucocyte numbers ($7.35x10^3$ cells/mm^3). From day 16/17 the leucocyte

numbers of animals in both groups begin to rise.

Detection of schizonts/piroplasms.

Examination of blood smears showed that macroschizont infected cells were detectable at day 15 in all six animals but the levels of parasite were low at approximately 0.1%. These levels remained the same until day 23 at which point schizonts were only perceptible in the blood of animal 13249. At day 24 no schizonts were detectable in the blood of any animals. Piroplasms were only detected in one animal (No. 13271/Group L) and only on one day (day 19), again the level of parasitemia was low at approximately 0.1%.

Fig. 5.1a, Temperature data from representative animals post immunisation with cells of clones I or L.

Temperature data (^{0}C) from animals 13274 (group I) and 13271 (group L) after immunisation with cells of either clone I or clone L.

Fig. 5.1b, PCV data from representative animals post immunisation with cells of clones I or L.

PCVs of animals 13274 (group I) and 13271 (group L) following immunisation with cells of clone I or clone L.

(All data presented relates to representative animals 13274 (immunised with clone I) and 13271 (immunised with clone L). Data relating to all six experimental animals is shown in the appendix).

Fig. 5.1a

Temperatures of animals immunised with either clone I or clone L.

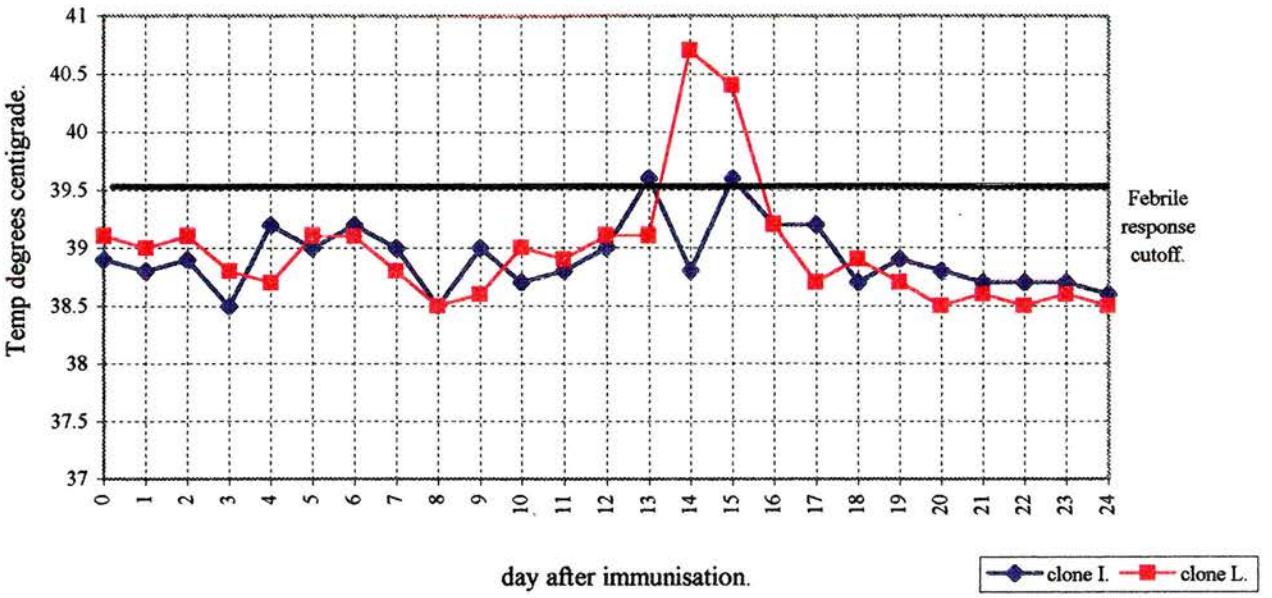


Fig. 5.1b

PCVs of animals from groups I and L.

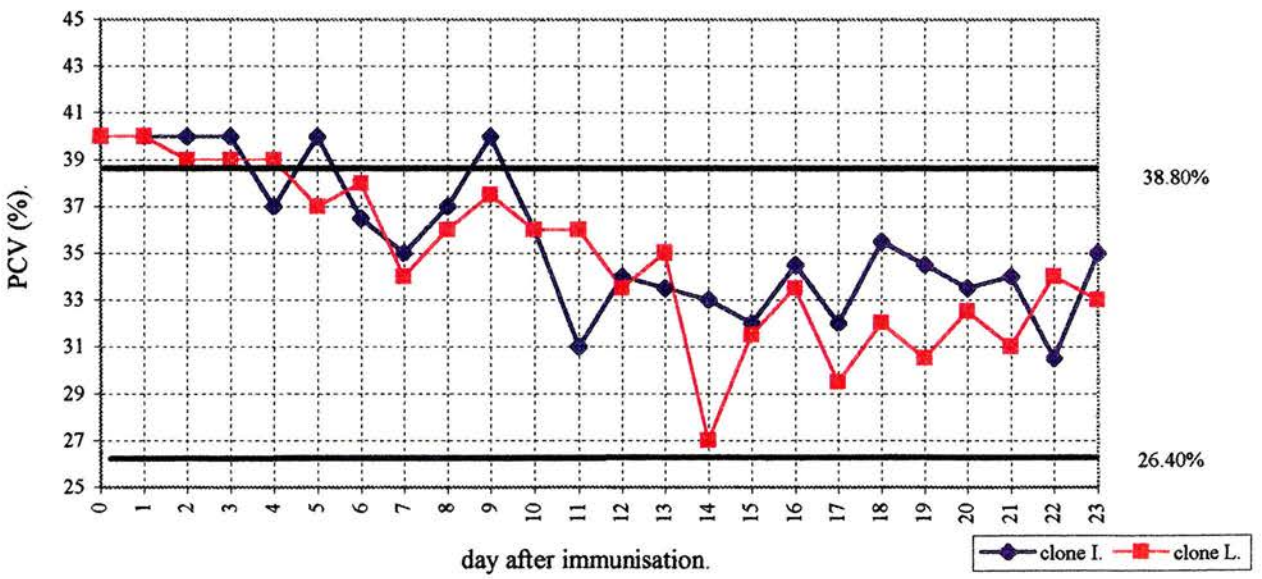


Fig. 5.1c, Total erythrocyte counts from representative animals after immunisation with either clone I or clone L

Total RBC count data after immunisation with cells of either clone I or clone L.

Fig.5.1d, Mean erythrocyte volume data from representative animals after immunisation with either clone I or clone L.

Mean RBC volumes after immunisation of animals with either clone I (13274) or L (13271).

Fig. 5.1c

Total erythrocyte counts from animals in groups I and L.

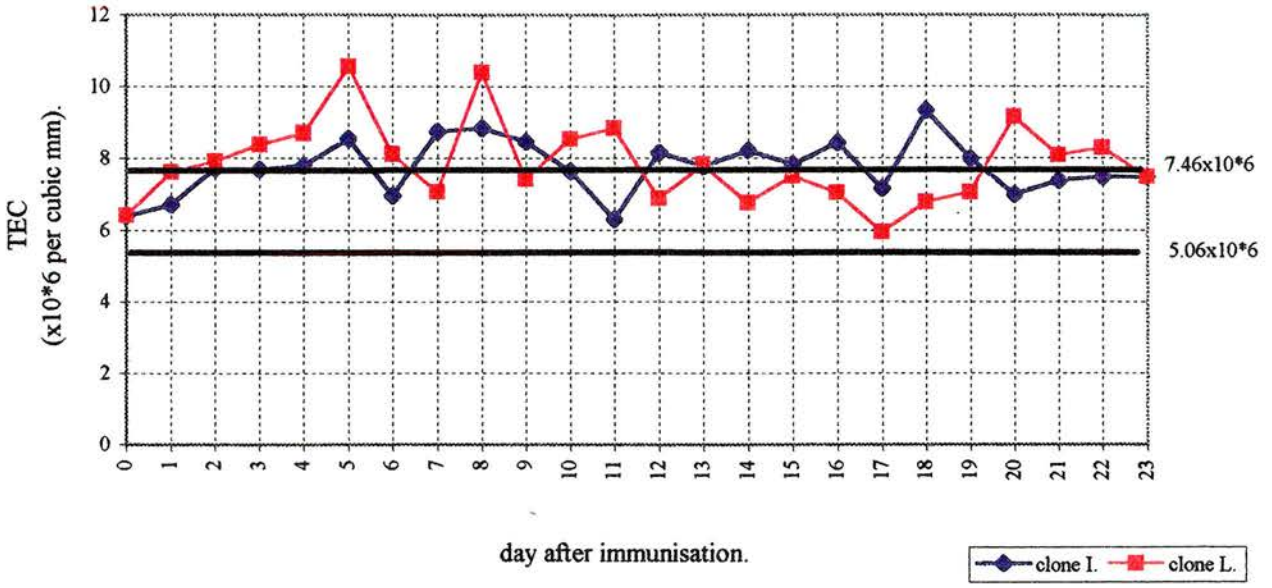


Fig. 5.1d

Mean RBC volumes of animals in groups I and L.

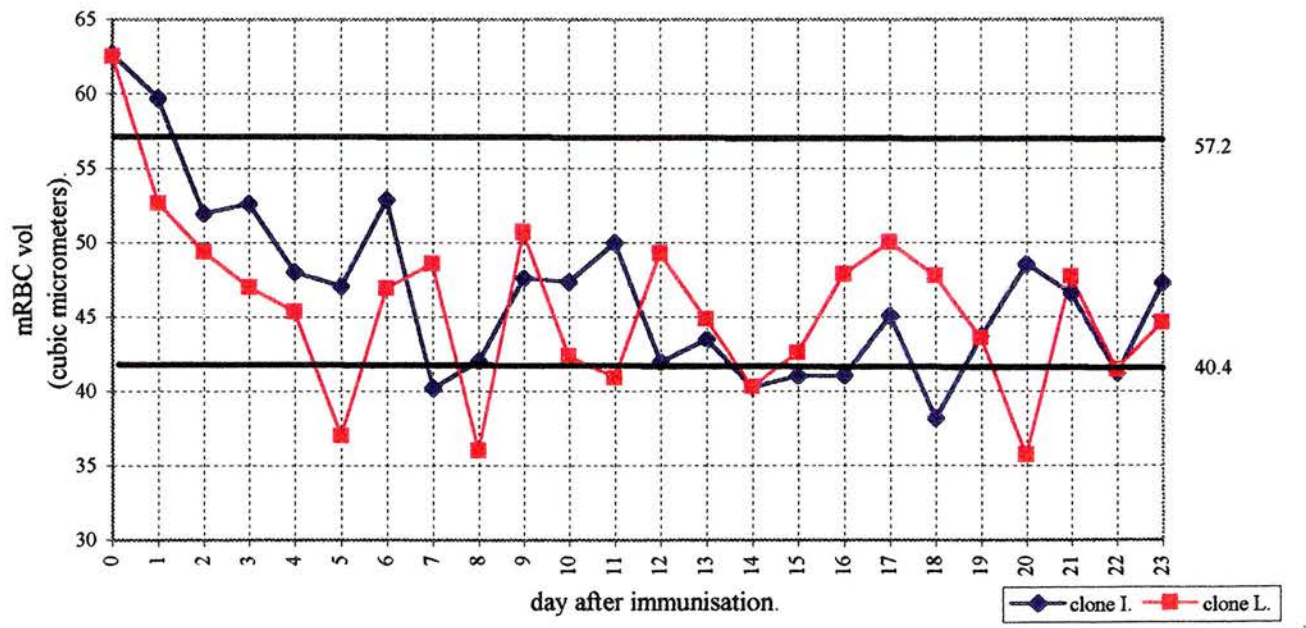
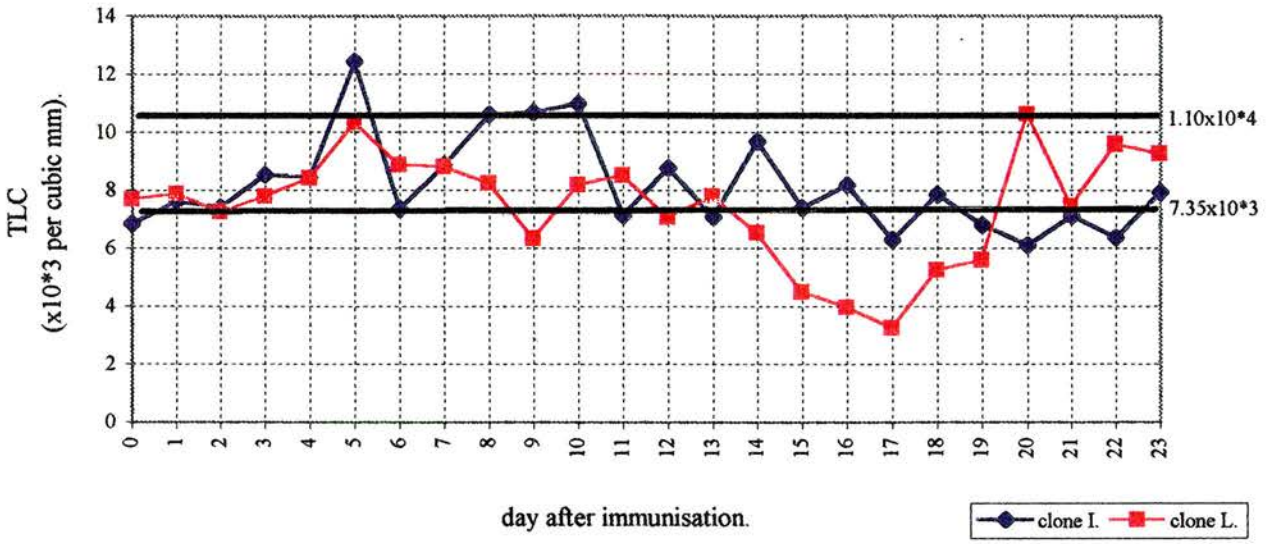


Fig.5.1e, **Total leucocyte count data from representative animals post immunisation with cells of either clone I or clone L.**

Data from animals 13274 (group I) and 13271 (group L) showing total leucocyte counts post immunisation.

Fig. 5.1e

Total leucocyte counts of animals from groups I and L.



Section 5.3.

Challenge trial.

Following a rest period of thirty days animals were challenged with an approximate LD50 dose of 1 TE of sporozoites (Gharb stock). Animals were assessed as before with temperatures and blood samples taken periodically over 20 days. Both groups I and L showed solid immunity to challenge and significant differences from the non-immunised control animals (Figs. 5.2a and 5.2b). Both control animals became ill. One animal 13050 became severely ill during this experiment, exhibiting classical signs of tropical theileriosis. These included, lethargy, loss of appetite, increase in temperature, frothing around the mouth and nostrils and a large fall in PCV. Due to the severity of these signs, a two day course of the antitheilerial Butalex was administered intramuscularly on days 9 and 10. During the following three days Butril (a veterinary antibiotic) was administered intravenously to guard against opportunistic infections.

Temperatures.

Temperature of the control animal (13050) increases rapidly after day 6 and only falls to the febrile response cut off point at day 15. Two group I animals exhibited transient increases in temperature (13254 on day 8 and 13274 on days 14/15 (40^0C/40.75^0C) (App 5.1.1). The animals of group L behaved in a similar fashion, with 13249 showing a fever on day 8 (39.5^0C) and 13266 a temperature of 40.0^0C on day 15 (App 5.1.2). From day 16 after challenge all six experimental animals, plus animal 13050 exhibited normal temperatures.

Animal 38B also showed an increase in temperature post challenge but did not exhibit as severe a fever as animal 13050 (App 5.1.3). The body temperature of 38B peaked at 40.75^0C on day 10, however, following this day the temperature begins to fall back towards normal levels. Animal 38B did not require Butalex treatment.

PCV analysis.

Following the challenge, the PCVs of the six animals from groups I and L vary between 30 to 35% and 27 to 35% respectively over the 20 days of the trial (Fig. 5.2b, App 5.1.4/5.1.5), all staying within the normal PCV range of a three year old heifer (38.8%-26.4%). The PCV of naive control animal 13050 fell from 35% to 24%

over the first 12 days, with the animal treated with Butalex on day 9. After this animal 13050 began to recover after day 14, reaching 32% by day 20 (Fig. 5.2b). The data collected from the second negative control animal 38B is unfortunately incomplete but a general trend can be seen (App 5.1.6). At the start of the challenge this animal had a PCV of 29%, half way through the trial this had decreased to 21% and on the final day of sampling shown was 17%. The PCV of this animal remained low (17% on day 28) but the animal did recover naturally over the following 14 days.

Total erythrocyte analysis.

Investigation of the TECs of all six vaccinated animals show that the circulating RBC pool of each animal is generally unaffected by sporozoite challenge (Fig 5.2c, App 5.1.7/5.1.8). This is not so for the naive control animals, animal 13050 exhibits wide fluctuations in TECs over the 20 days of monitoring. An initial rise in the numbers of circulating RBCs can be seen over the first 5 days after challenge, which is followed by a fall in numbers until day 12 (post treatment), after which the numbers of RBCs begin to increase and pass that observed at the beginning of the trial.
Again complete data for animal 38B is unavailable but the general trend discernible would suggest that the pattern of fluctuation in the numbers of circulating RBCs over the first 10 to 12 days of the trial are similar to those observed in animal 13050. After day 12 the numbers of RBCs continue to fall and remain low until the end of the monitoring.

Mean volumes of circulating RBCs.

Mean RBC volumes of the immunised animals remained generally unaffected by challenge, with the average erythrocytic volumes remaining within normal limits (40.4-57.2μm^3) (Fig 5.2d, App 5.2.0/5.2.1). The control animals behaved very differently from the immunised animals. Animal 13050 began the trial with a mRBC volume of 57μm^3, this fell rapidly over the next 5 days and showed a large degree of fluctuation until day 11, after which the mean volume fell to well below the lower limit for a healthy animal (40.4μm^3) (Fig. 5.2d). No. 38B exhibited an initially low mRBC volume of 38.5μm^3 which fluctuated between 35.5 and 40μm^3 over first 14 days of the trial. From this point erythrocyte volume increased, peaking on day 19 at

$42.5\mu m^3$ (App 5.2.2).

Fig. 5.2a, Temperature data from representative animals following challenge with 1 TE of Gharb sporozoites.

Temperature data (^{0}C) collected post challenge of control animals and animals preimmunised with $1x10^6$ cells of either clone I or clone L, with 1 TE of sporozoites.

Fig. 5.2b, PCV data from representative animals following challenge with 1 TE of Gharb sporozoites.

PCV data collected post challenge of control animals and animals preimmunised with either $1x10^6$ cells of clone I or clone L, with 1 TE of sporozoites.

(Data from representative animals in groups I and L again collected from animals 13274 and 13271, control data shown collected from animal 13050).

Fig. 5.2a

Temperatures of animals challenged with sporozoites.

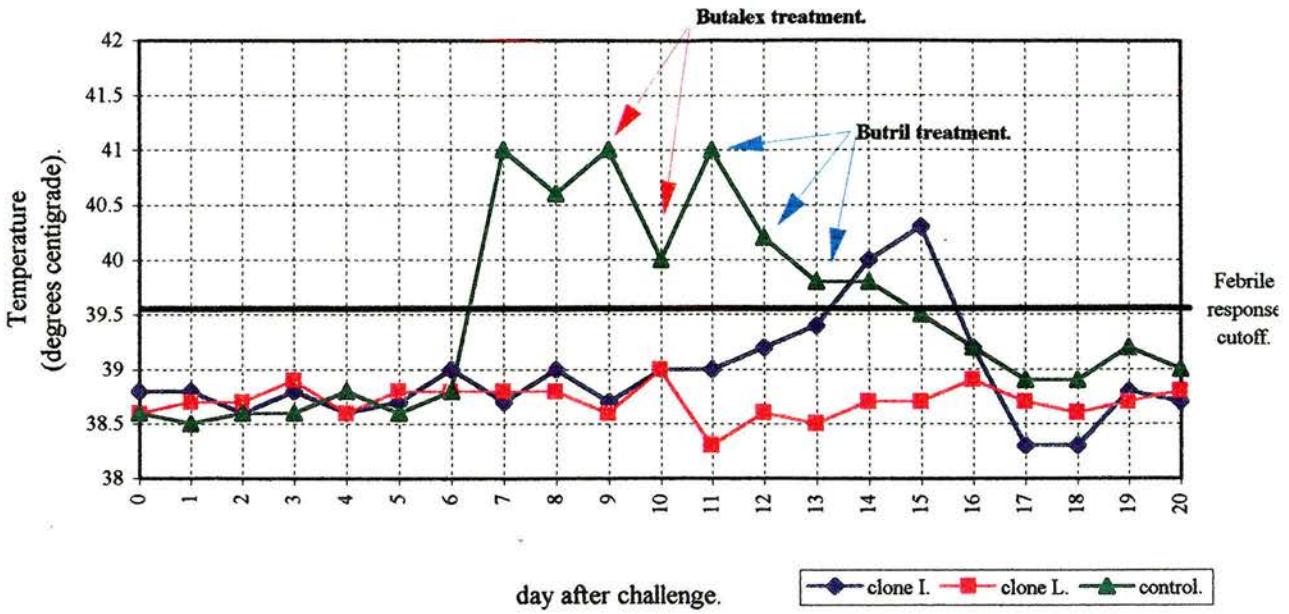


Fig. 5.2b

PCVs of animals after challenge with sporozoites.

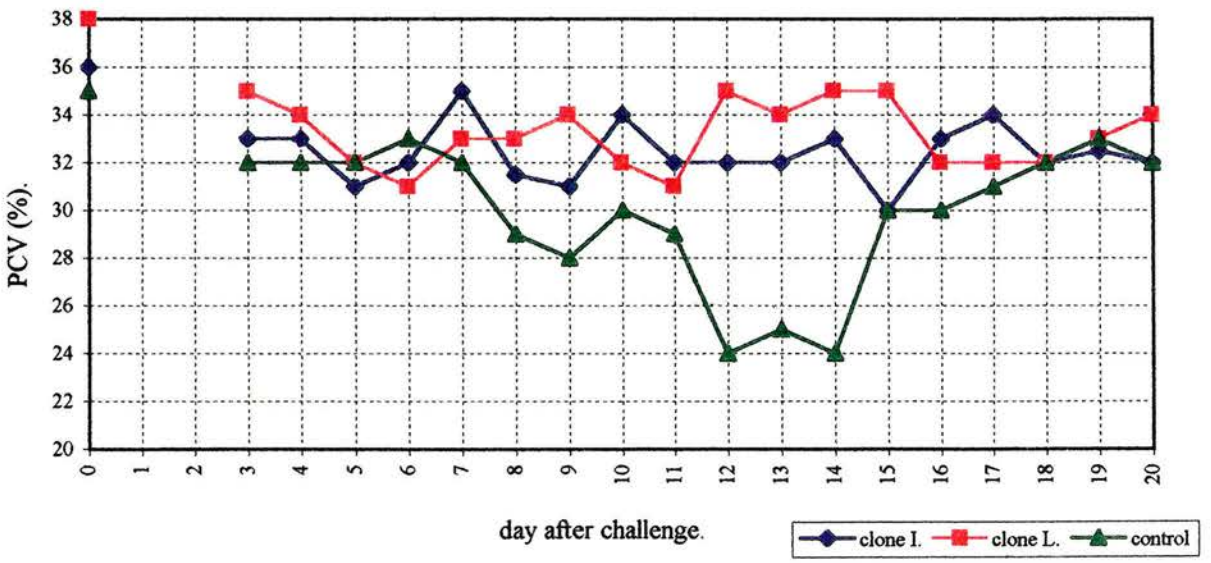


Fig. 5.2c, Total erythrocyte count data from representative animals following challenge with 1 TE of Gharb sporozoites.

Total erythrocyte count data collected from animals immunised with $1x10^6$ cells of either clone I or L and control animal 13050 after challenge with 1 TE of sporozoites.

Fig.5.2d, Mean erythrocyte volume data from representative animals following challenge with 1 TE of Gharb sporozoites.

Mean RBC volume data collected from control animal 13050 and animals preimmunised with $1x10^6$ cells of either clone I or clone L following challenge with 1 TE of Gharb sporozoites.

Fig. 5.2c

Total erythrocyte counts of animals challenged with sporozoites.

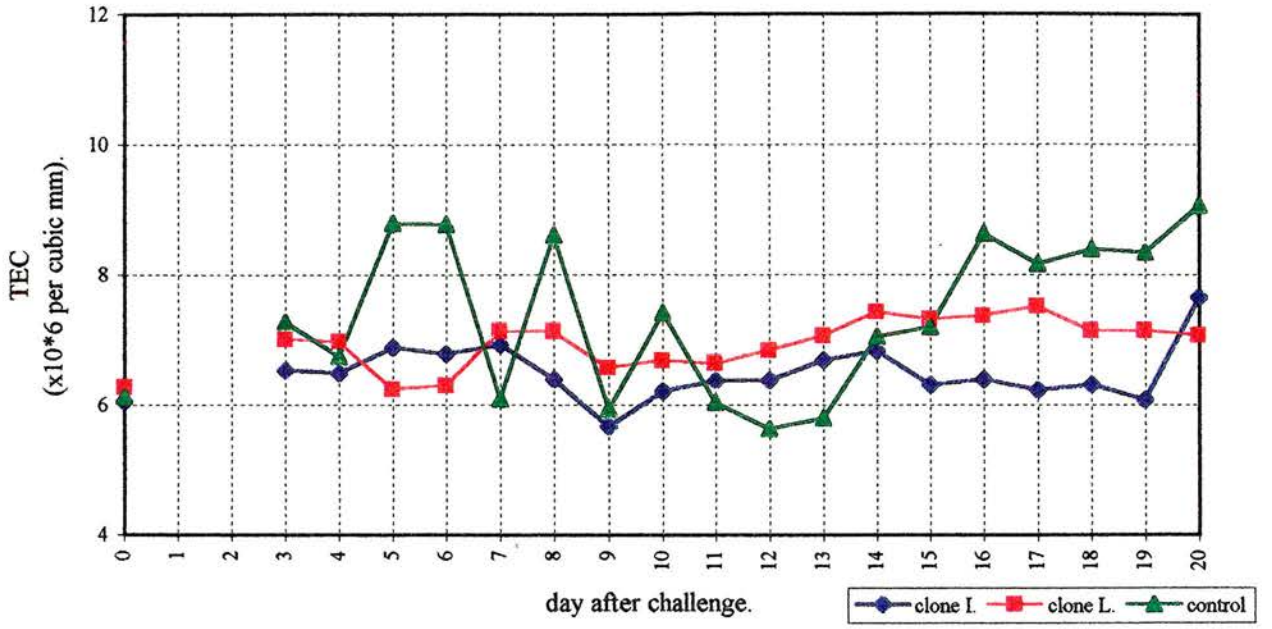
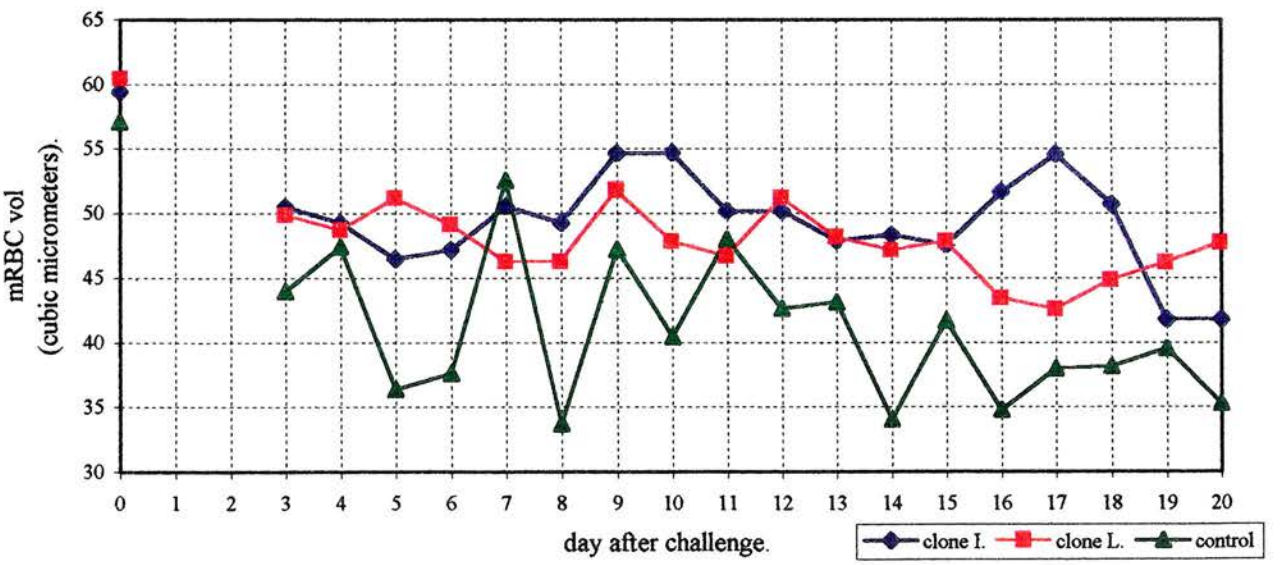


Fig. 5.2d

Mean RBC volumes of animals after challenge with sporozoites.



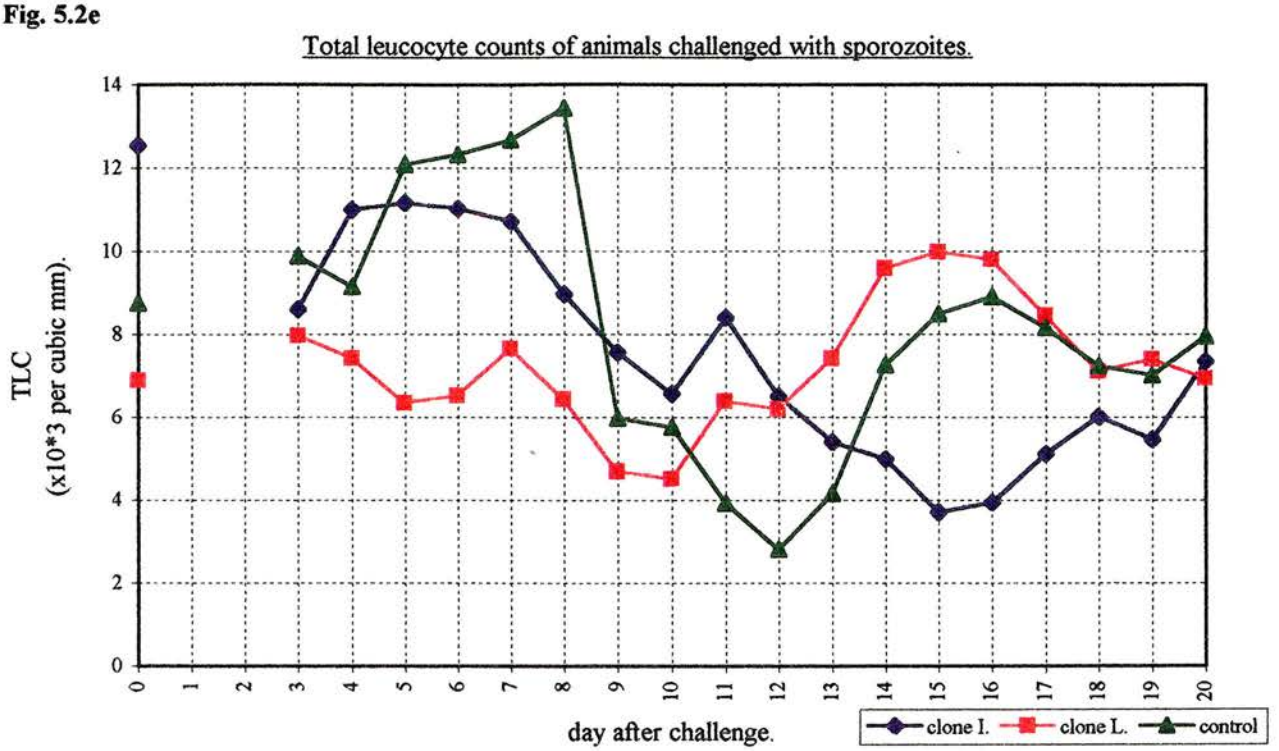
Total leucocyte analysis.

There are large fluctuations in the numbers of leucocytes present in the periphery of immunised animals following challenge. All six animals show levels which drop below normal ($7.35x10^3$cell/mm^3), indicated by the representative data shown in Fig 5.2e. There would appear to be little difference in the severity of leucocyte fluctuations of animals immunised with either clone I or clone L (App 5.2.3/5.2.4). The TLCs of the control animal 13050 show marked variations over the 20 days of the trial (Fig. 5.2e). An initial rise in TLC over the first 8 days of the trial was followed by a dramatic fall of $1.1x10^4$ cell/mm^3 in leucocyte number between days 8 to 12. After this large reduction in the circulating leucocyte pool the number begins to rise over the next 8 days and by day 20 approaches the lower normal limit of this parameter. The second control animal (No. 38B) showed a TLC profile which fitted roughly in to that of animal No. 13050 (App 5.2.5). This is to say there was an initial increase in leucocyte number, followed by a large decrease and subsequent rise back towards normal levels towards the end of the trial. However, the variations in leucocyte numbers by 38B are not as severe as those observed in 13050.

Fig. 5.2e, Total leucocyte count data from representative animals following challenge with 1 TE of Gharb sporozoites.

Total leucocyte data collected from control animal 13050 and animals preimmunised with $1x10^6$ cells of either clone I or clone L after challenge with 1 TE of Gharb sporozoites.

Fig. 5.2e


Total leucocyte counts of animals challenged with sporozoites.
TLC
(x10*3 per cubic mm).
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
day after challenge.
clone I.
clone L.
control

Detection of schizonts and piroplasms post challenge.

Following challenge of the six original animals and two naive controls, blood smears and cytospin preparations of PBM were made every day. No schizonts or piroplasms were detected in any of the immunised animals. Control animal 13050 became very sick following infection, with fever starting between days 6-7. This animal was treated with Butalex on day 9 and 10. Animal 38B was not treated and data shows that this animal suffered a fairly high parasitemia. Schizonts were first detected on day 6 and piroplasms on day 11. By day 14, No. 38B showed a parasitemia of 13.1% and by day 19 this had risen to 43.7%. This parasitemia remained high until the end of the trial (*i.e.* 19% - day 28), however, the infection did was cleared. whilst the trial ran No.38B also showed high levels of micro and macroschizonts present in the periphery between days 7 - 24. This data showing that the 1 TE challenge of *T.annulata* (Gharb) 0sporozoites was capable of causing all the clinical signs of tropical theileriosis.

Analysis of infected cells isolated from immunised animals.

After macroschizont infected cells are inoculated into naive animals parasite material is known to transfer from donor cells into cells of the recipient (Innes, 1989; Nichani, 1994). It is also known that parasite transfer from donor to recipient cells is necessary for the production of immunity (Nichani *et al*, 1997). Transfer of macroschizont material from one cell type to another, may result in changes in the behaviour of the parasite/infected cell population, which in turn may affect animals post vaccination (*i.e.* induce fever). To assess whether this is the case infected cells were isolated from animals post immunisation and analysed to ascertain if parasite had crossed over from donor to recipient cells. Also to ascertain if this process had altered the expression of cytokine mRNAs by infected cells from those expressed by clones I and L.

MHC class I, II and genotype analysis.

Before macroschizont infected cells of clone I or L (produced from the same animal) were administered to the experimental animals their MHC class II types were investigated by polymerase chain reaction - restriction fragment length polymorphism analysis (PCR-RFLP). This analysis was kindly performed by Robert Oliver of the Roslin Institute. Table 5.2 contains the MHC class II types of the immunising cell lines and the infected cells isolated from the recipient animals after four weeks in culture.

Table 5.2.

Animal No/cell line.	*MHC class II DRβ_3 type.*
T.a 12929 (clone I).	14, unknown.
T.a 12929 (clone L).	14, unknown.
T.a 13274 (immunised with clone I).	1, 22.
T.a 13254 (immunised with clone I).	7, 27.
T.a 13260 (immunised with clone I).	7, 7 (homozygote).
T.a 13266 (immunised with clone L).	1, 18.
T.a 13271 (immunised with clone L).	8/9, 10.
T.a 13249 (immunised with clone L).	12, 14.

Table 5.2 MHC class II types of experimental animals determined by PCR-RFLP.

MHC class II types of the original infected clonal lines (I and L) and those of the cell lines isolated from the six experimental animals after immunisation. Only one of the newly isolated cell lines (T.a 13249) shares an MHC class II allele with the clonal lines I and L.

Of the six experimental animals shown in Table 5.2 only one (No. 13249) shares an MHC class II allele with the immunising cell lines (DRβ_3 type 14). MHC class I type analysis and microsatallite analysis were also performed on these eight cell lines. It was found that the MHC class I types of clones I and L did not match those of macroschizont infected cell lines isolated after immunisation (micocytotoxicity MHC class I typing tests were performed by Angela Gallagher of the Roslin Institute). Also infected cells isolated from the experimental animals differed from clone I and L with respect to five different bovine microsatellite markers. This work (kindly performed by Janice Barr), investigated the sequences of five different microsatillite

markers in each of the eight cell lines. Clones I and L contained the same satellite sequences but those of the six newly isolated cell lines did not match those of clones I and L. These three sets of data therefore show that the infected cells isolated from the six experimental animals do not originate from animal 12929. The parasite contained in cells of clone I and clone L has therefore passed from the immunising cell lines, to infect cells of the recipient animals.

FC analysis of infected cells isolated post immunisation.

FC analysis was performed upon the six macroschizont infected cells lines produced from the infected cells isolated after immunisation. The data obtained from this analysis is presented in Fig 5.3a (I-iv) to Fig 5.6b (I-iv). The cell lines were investigated for expression of the epitopes detailed in chapter III, section 3.3 (MHC class I/II (Dutia *et al*, 93; 95, CD14 (Gupta *et al*, 96) and the epitope bound by IL-A24 (Ellis *et al*, 87)).

MHC class I & II expression.

The level of expression of class I expressed by clones I and L are shown in Figs 5.3a (I) and Fig 5.3b (I) respectively. The expression of these molecules by the clonal lines is very similar with a narrow range in class I expression (X-axis). All the newly isolated cell lines again show high expression of MHC class I molecules (possibly at higher levels than the original clones) but the ranges in expression have increased following cross over into the recipient cells.

MHC class II expression of the six newly isolated lines is also different from the cells used to immunise the experimental animals. Of the two clonal lines, clone I expressed higher levels of class II molecules, than clone L (Fig 5.4a (I)). However, one can see that infected cells isolated from immunised animals, express much greater ranges than did the original clones (I & L). Clone L expressed very low levels of MHC class II molecules but this is not the case for the newly isolated infected cells. Data contained in Fig 5.4b (I) shows that class II expression by this clone to be much lower than the levels expressed by the lines produced from animals 13266, 13271 and 13249 (Fig 5.4b (ii-iv)).

Fig 5.3a **FC histograms of MHC class I molecule expression by cells of clone I and infected cell lines derived from animals immunised with clone I.**

(i) MHC class I expression of clone I, (ii-iv) MHC class I expression of cell lines isolated from animals (13274, 13254 & 13260) immunised with clone I. Negative control profiles are shown in blue and profiles resulting from labelling with the anti class I mAb IL-A19 are shown in red.

Fig 5.3b **FC histograms of MHC class I molecule expression by cells of clone L and infected cell lines derived from animals immunised with clone L.**

(i) MHC class I expression of clone L, (ii-iv) MHC class I expression of cell lines isolated from animals (13266, 13271 & 13249) immunised with clone L. Negative control profiles are shown in blue and profiles resulting from labelling with the anti class I mAb IL-A19 are shown in red.

Fig 5.3a

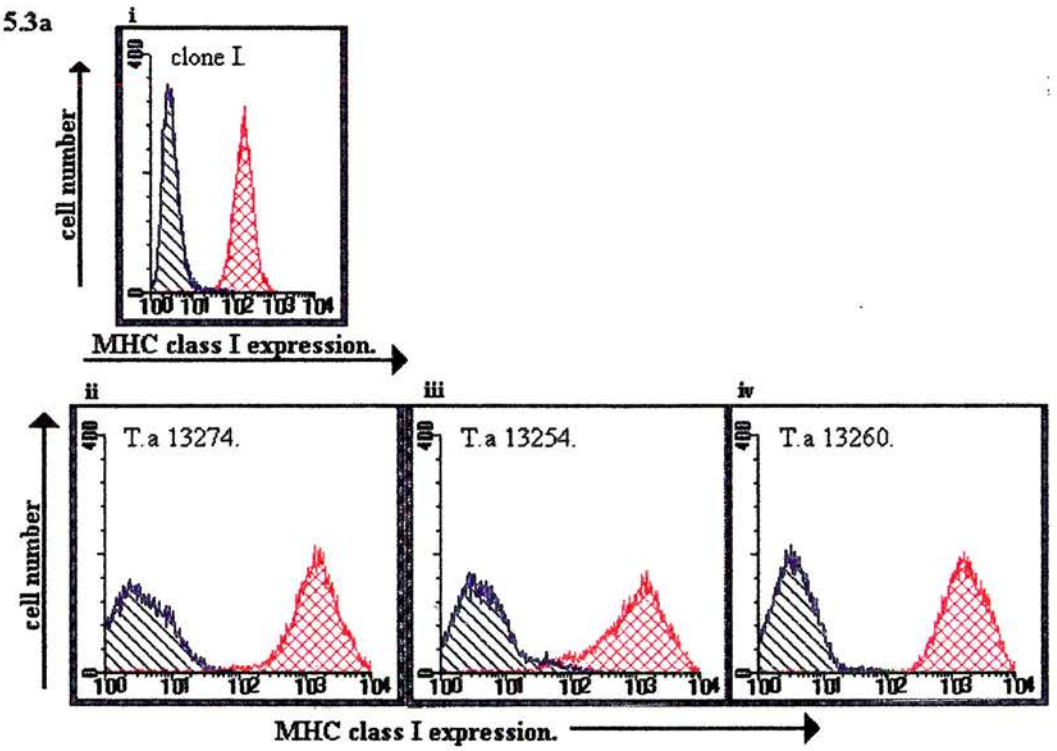


Fig 5.3b

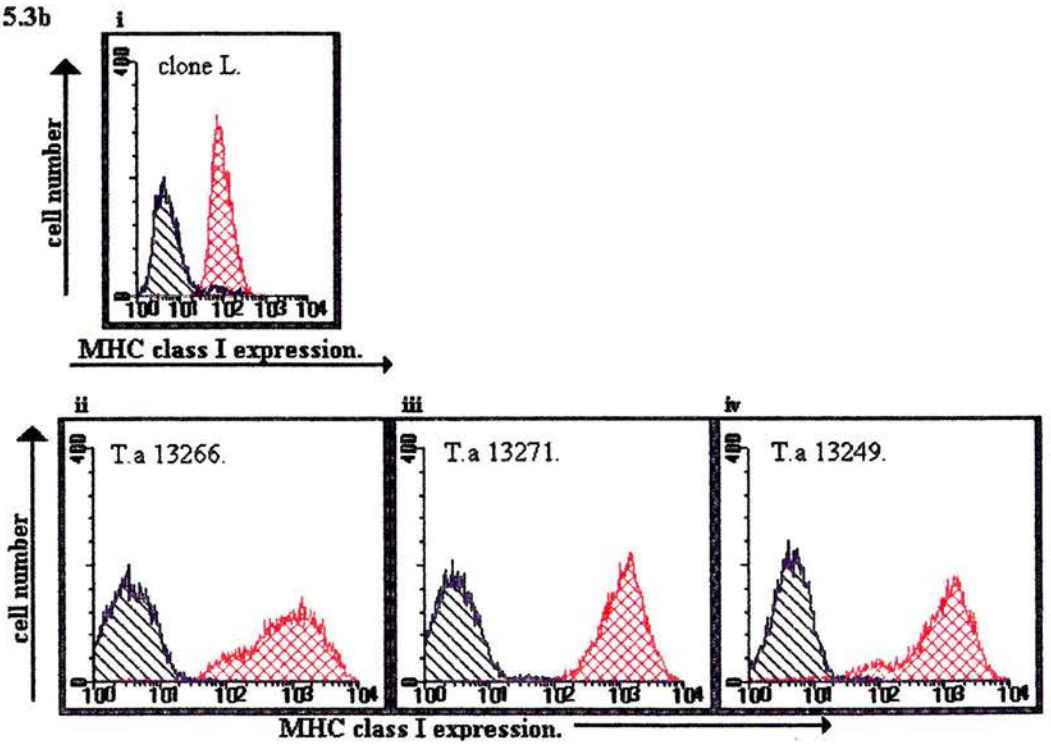


Fig 5.4a **FC histograms of MHC class II molecule expression by cells of clone I and infected cell lines derived from animals immunised with clone I.**

(i) MHC class II expression of clone I, (ii-iv) MHC class II expression by cell lines isolated from animals (13274, 13254 & 13260) immunised with clone I. Negative control profiles are shown in blue and profiles resulting from labelling with the anti class II mAb IL-A21 are shown in red.

Fig. 5.4b **FC histograms of MHC class II molecule expression by cells of clone L and infected cell lines derived from animals immunised with clone L.**

(i) MHC class II expression of clone L, (ii-iv) MHC class II expression cell lines isolated from animals (13266, 13271 & 13249) immunised with clone L. Negative control profiles are shown in blue and profiles resulting from labelling with the anti class II mAb IL-A21 are shown in red.

Fig 5.4a

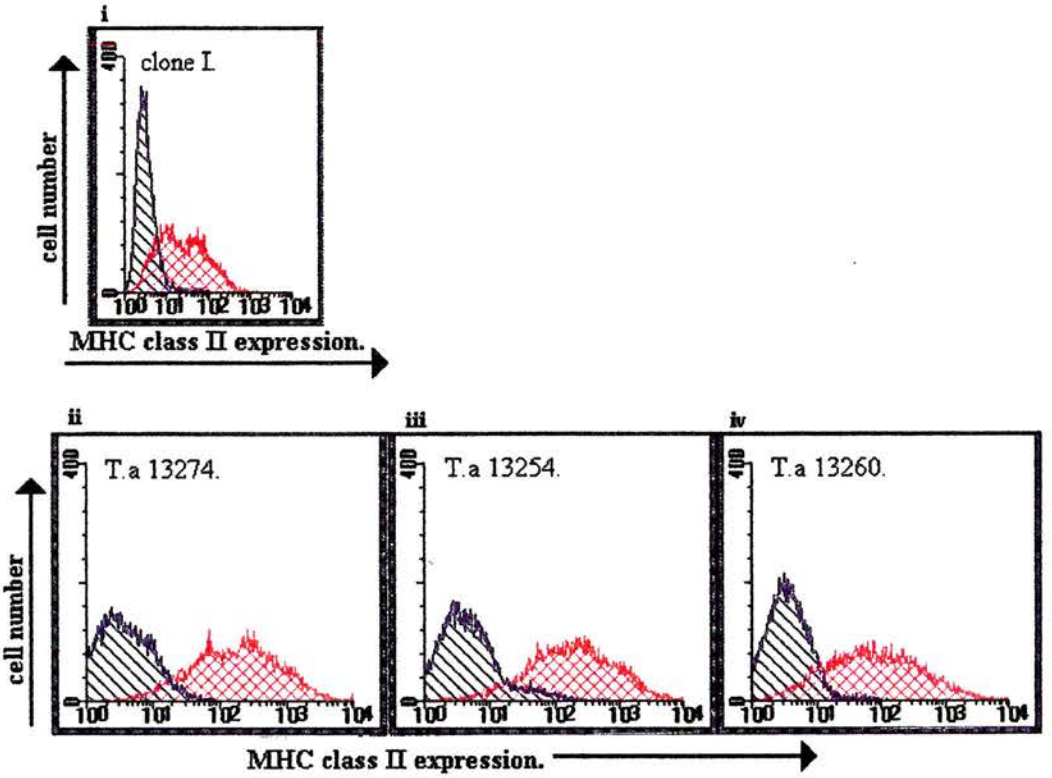
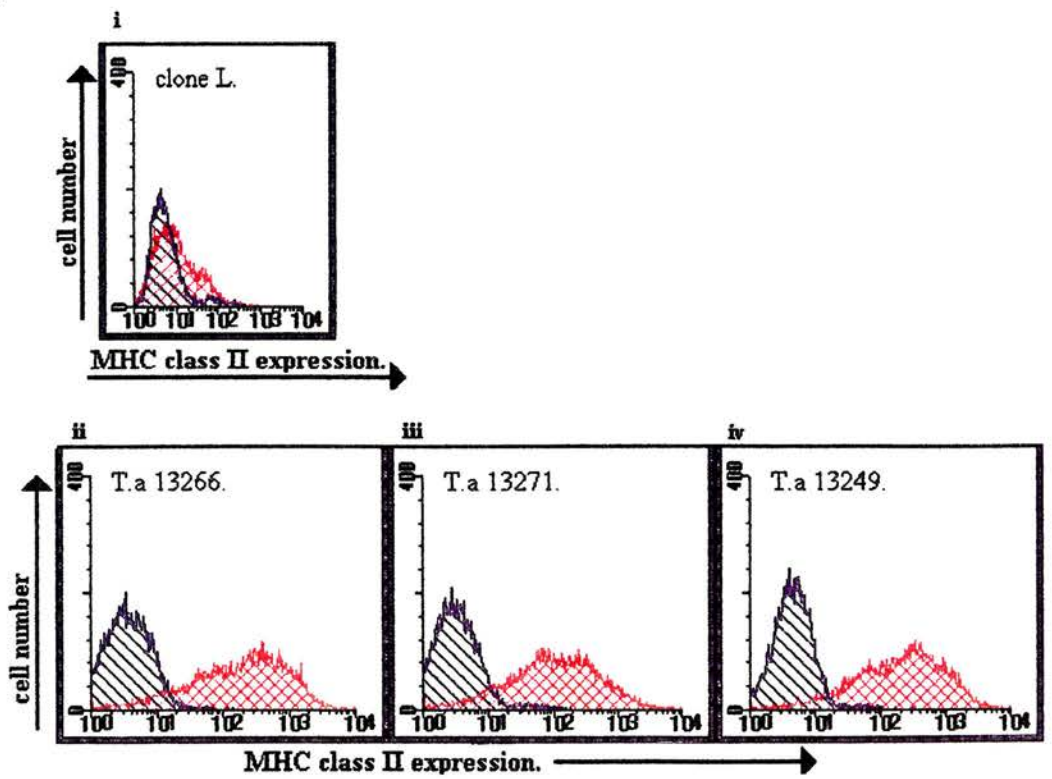


Fig 5.4b



CD14 expression by cells isolated post immunisation.

Data presented in chapter III showed that after infection of $CD14^+$ cells by *T.annulata* the expression of CD14 is drastically down regulated (Figs 5.5a (I) and 5.5b (I)). This is also the case for the infected cells isolated from five out of six of the experimental animals. The infected cells isolated from animals immunised with clone I all exhibit low levels of CD14 expression, similar to that of the immunising clonal line. Of the three cell lines produced after immunisation of animals with clone L, two express similar levels of CD14 to the original population (Fig 5.5b (iii,iv) but those produced from infection of animal 13266 express slightly higher levels of this molecule (Fig 5.5b (ii)).

Expression of the epitope bound by IL-A24.

Both clones I and L express the epitope bound by IL-A24, however, not all of the newly isolated cells from the recipient animals expresses this epitope to a similar degree. Of the six macroschizont infected cell lines produced from the experimental animals, four show levels of expression similar to those of the immunising clones. However, two cell lines produced from animals immunised with clone I exhibited levels of expression markedly lower than that of the immunising cell line (Fig 5.6a (ii, iii).

Fig. 5.5a **FC histograms of CD14 expression by cells of clone I and infected cell lines derived from animals immunised with clone I.**

(i) CD14 expression of clone I, (ii-iv) CD14 expression cell lines isolated from animals (13274, 13254 & 13260) immunised with clone I. Negative control profiles are shown in blue and profiles resulting from labelling with the anti CD14 mAb VPM65 are shown in red.

Fig. 5.5b **FC histograms of CD14 expression by cells of clone L and infected cell lines derived from animals immunised with clone L.**

(i) CD14 expression of clone L, (ii-iv) CD14 expression cell lines isolated from animals (13266, 13271 & 13249) immunised with clone L. Negative control profiles are shown in blue and profiles resulting from labelling with the anti CD14 mAb VPM65 are shown in red.

Fig 5.5a

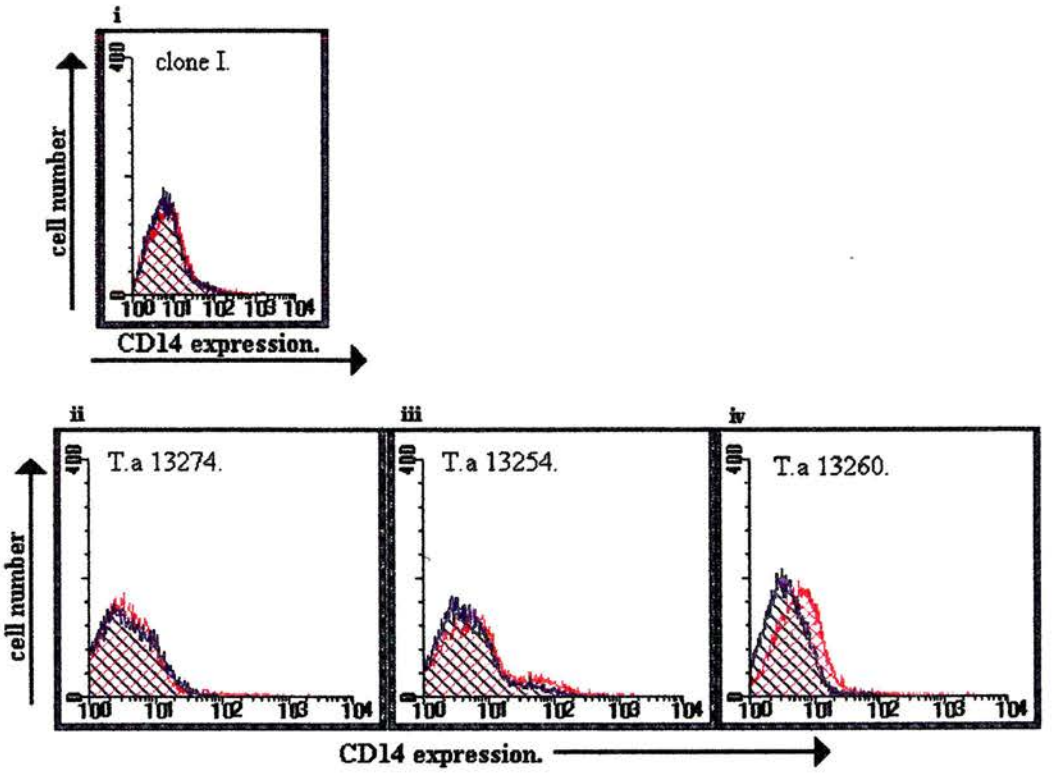


Fig 5.5b

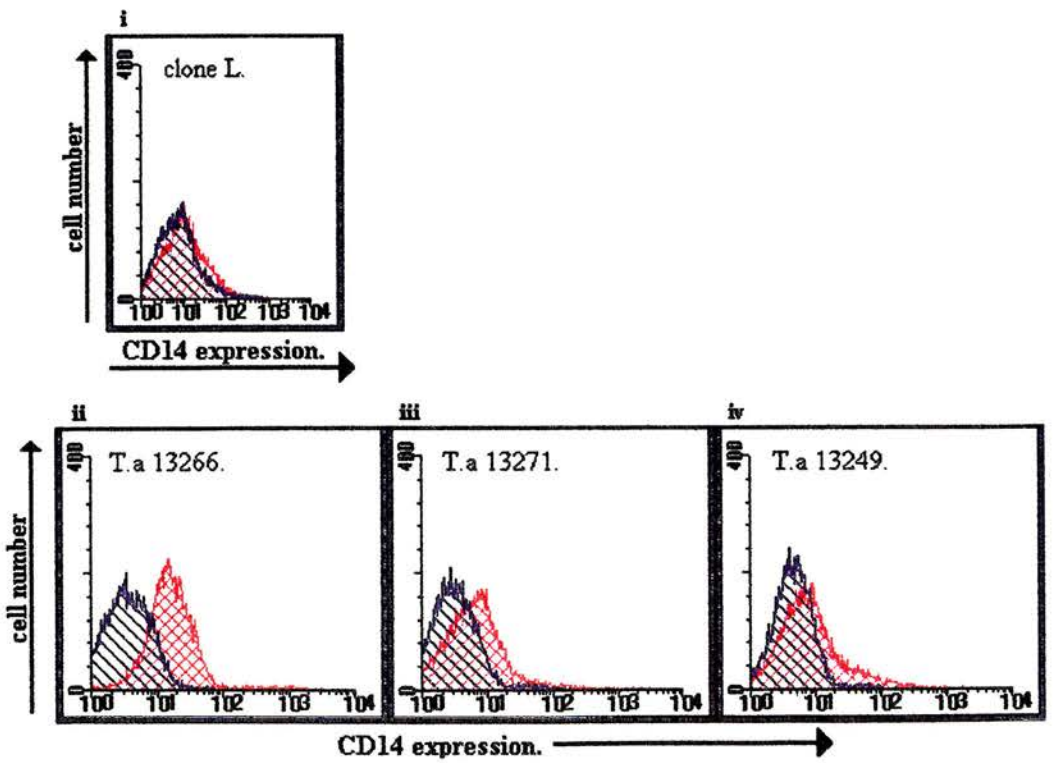


Fig 5.6a **FC histograms of expression of the epitope bound by mAb IL-A24 by cells of clone I and infected cell lines derived from animals immunised with clone I.**

(i) Expression of the epitope bound by IL-A24 by clone I, (ii-iv) Expression of the epitope bound by IL-A24 by cell lines isolated from animals (13274, 13254 & 13260) immunised with clone I. Negative control profiles are shown in blue and profiles resulting from labelling with IL-A24 are shown in red.

Fig 5.6b **FC histograms of expression of the epitope bound by mAb IL-A24 by cells of clone L and infected cell lines derived from animals immunised with clone L.**

(i) Expression of the epitope bound by IL-A24 by clone L, (ii-iv) Expression of the epitope bound by IL-A24 by cell lines isolated from animals (13266, 13271 & 13249) immunised with clone L. Negative control profiles are shown in blue and profiles resulting from labelling with IL-A24 are shown in red.

Fig 5.6a

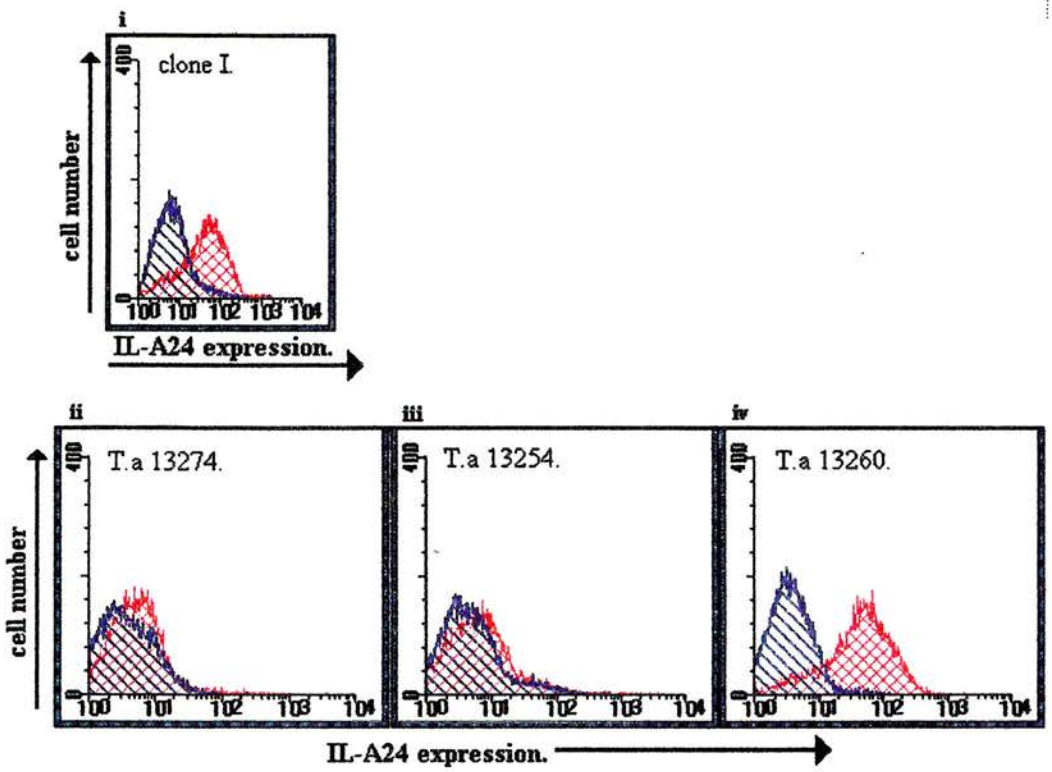


Fig 5.6b

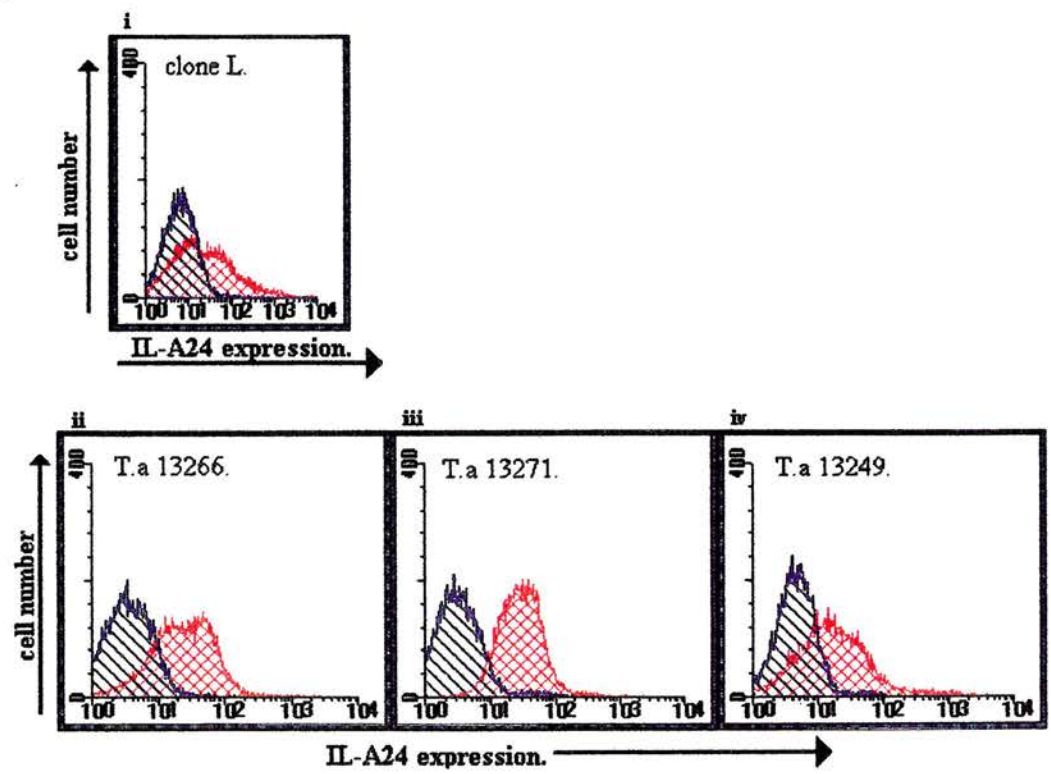


Table 5.3

	Isolated cell lines					
	Immunising cells - clone I.			*Immunising cells - clone L.*		
Products	T.a 13274	T.a 13254	T.a 13260	T.a 13266	T.a 13271	T.a 13249
G3PD	20	--	20	20	20	20
IL-1α	20	--	22	20	20	24
IL-1β	20	--	20	20	20	22
IL-6	20	--	20	20	22	24
IL-10	N.P.	--	N.P.	N.P.	N.P.	N.P.
TNFα	24	--	22	20	24	N.P.

Table 5.3 Preliminary limiting cycle RT-PCR analysis of cytokine mRNA production by lines isolated from animals immunised with either clone I or L.

Semi-quantitative RT-PCR analysis of cytokine mRNA production by macroschizont infected cells isolated from five of the six experimental animals. RT-PCR procedures used a constant 5μg of total RNA and 2μl of cDNA, followed by between 20 to 30 amplification cycles (increasing in step of 2). N.P.= PCR products not present at 30 cycles, data from cell line T.a 13254 unavailable.

The data contained in table 5.3 above were produced during preliminary limiting cycle RT-PCR analysis of the cytokine mRNA production of cell lines isolated on day 15 after immunisation of the six experimental animals. Unfortunately, data was not obtained from cell line 13254. However, the data obtained from the other lines does show that the production of the various cytokine mRNAs by the five other lines is

remarkably similar, regardless of whether the lines were isolated from an animal immunised with clone I or clone L.

Discussion.

Section 5.5.

Introduction.

Vaccination with long term cell culture attenuated macroschizont infected cell lines is currently the most effective control method for tropical theileriosis. However, the production of these cell lines is problematic. Amongst these problems are: (1) cells used to produce the putative vaccines are often poorly characterised, (2) production and maintenance of the vaccine lines incurs high costs, (3) attenuation of infected cells requires between 2 to 3 years of tissue culture and the mechanisms of attenuation are poorly understood. Investigations which elucidate the mechanisms of attenuation and shorten the production time of new vaccines would be extremely advantageous and desirable.

It has been suggested that it is the macroschizont stage of *T.annulata* infections which are responsible for the majority of pathology associated with this disease (Hooshmand-Rad, 1976). Indeed instances have been documented where animals died before any piroplasms have been detected in the periphery, underlining the fact that the macroschizont stage is capable of producing illness and pathology (Hooshmand-Rad, 1976). Also the characteristics of pathology associated with tropical theileriosis, such as fever, anaemia, inflammation, cachexia and inappropriate T cell activation may be linked to the activities of infected cells, such as the possible production of inflammatory and T cell stimulatory cytokines (Neitz, 1957; Barnett, 1977; Waage *et al*, 1989; Campbell *et al*, 1995). The post vaccinal reactions observed often include fever and anemia (Pipano, 1981) and the occurrance of these signs may be linked to the T cell stimulatory ability and proinflammatory cytokine production by infected cells.

The isolation of clones I and L from a cell line of known phenotype (as opposed to a cell line obtained by infecting whole PBM), which showed marked differences with respect to cytokine mRNA production and T cell stimulatory ability, provided an

unique opportunity to investigate the relationship between cytokine production/T cell stimulation and the induction of pathology by the macroschizont stage of this parasite. The stimulation of T cells by *T.annulata* infected cells causes the production of exceptionally high levels of IFNγ (Campbell *et al*, 97). Campbell *et al* (1997) showed efferent lymph leaving a lymph node containing infected cells could contain levels of IFNγ upto 20x those observed in a protective immune response. INFγ from T cells and other cytokines thought to be produced by infected cells (IL-1, IL-6 and TNFα) are proinflammatory and can act in cascade mechanisms, leading to further production of proinflammatory cytokines and other molecules such as prostaglandins (Waage *et al*, 1989; Creasey *et al*, 1991). The production of cytokines by infected cells and infected cell stimulated T cells may play an important role in *T.annulata* induced pathology.

Section 5.6.

Post vaccinal reactions induced by clones I & L.

The severity of signs observed post immunisation with clones I and L did depend upon which clone the animals received. Clone I was shown to induce low levels of *in vitro* autologous T cell proliferation and also low levels of T cell stimulatory/inflammatory cytokine mRNA species. Animals which received cells of clone I experienced only mild fevers and fluctuations in PCV and TLCs. However, animals which received cells of clone L, shown to induce higher levels of T cell proliferation in autologous T cells and produce higher levels of cytokine mRNA species exhibited significantly higher levels of fever and significantly greater falls in PCV and leucocyte numbers. Therefore the reactions observed post immunisation correlate with the data obtained from the T cell proliferation and limiting cycle RT-PCR analysis of these clones.

Section 5.7.

Protection induced by immunisation with clones I & L.

Challenge of cattle after immunisation with these clonal lines showed that both lines were able to vaccinate against a potentially lethal dose of sporozoites. The *T.annulata* Gharb sporozoite stabilate provided by Professor Brown (C.TV.M) had been shown

to produce an LD50 (at a dose of 1 TE) (Nichani, 1994) and induced severe signs in both unimmunised control animals (animals 13050 and 38B). Animal 13050 required Butalex treatment at day 9 post challenge. Animal 38B also showed signs classically associated with tropical theileriosis and took approximately two months to recover from the challenge. However, the responses of all the six immunised animals were similar with only mild signs observed after inoculation with sporozoites.

A novel approach was used to select these putative clonal cell lines. Normally vaccine lines are produced by the selection of clonal parasite populations of low virulence by long term tissue culture. But long term tissue culture attenuation appears to act on the parasite and can lead to over-attenuated cell lines, which do not provide satisfactory protection, especially against heterologous challenge (Dargouth *et al*, 1996; Ilhan *et al*, 1997). The method of cloning I employed, coupled with assessment of T cell stimulatory ability and cytokine mRNA production of infected cells allowed the rapid selection of cell populations with attenuated phenotypes, which were capable of inducing strong protective immunity against a heterologous challenge.

A possible reason for the success of these two clonal lines as vaccines may be that infected populations were selected with respect to the characteristics of the infected cells and not the parasite they contained. This allowed the production of putative vaccine lines (after only a matter months of tissue cutlure), which remained highly immunogenic as they contain parasite unattenuated by long term tissue culture.

Section 5.8.

Isolation & characterisation of infected cells from animals prior to challenge.

Infected cells from each immunised animal were isolated and grown in tissue culture, forming new infected lines. MHC class I/class II and microsatellite analysis showed these infected cells to originate from the recipient animals and the donor. Thus showing that the macroschizont material has "crossed over" to infect cells of the recipient. FC analysis of the new cultures showed them to differ greatly from the original infected cell populations. MHC class I and II expression of all the six newly isolated lines was shown to be higher than the original clones. Also these molecules showed a greater range of expression within the new populations, suggesting that the newly isolated lines are not clonal in origin.

MHC class I expression.

MHC class I expression can play a crucial role in vaccination against *T.annulata* infection. Nichani (1994) showed that if animals had been previously exposed to the MHC class I type of a vaccine line subsequently used to immunise them, then no immunity was induced following immunisation. The reason for this remains unclear but is probably connected to the fact that pre-immunisation with cells of a certain MHC type will sensitise animals to the presence of those cells/MHC types. This being the case immunising cells will be removed quickly, as a graft would be if an animal had already received a graft from the same source. The rapid removal of the immunising cell line before infected cells have established themselves within the recipient, presumably inhibits the production of a protective immune response (Pipano, 1993). The use of soft agar cloning and selection of attenuated cell lines should allow the rapid production of new lines. If this is the case then it will be possible to make numerous cell line vaccines, avoiding the problem of revaccinating animals with the same cell lines.

MHC class II & CD14 expression.

There are also differences between the original lines (clones I & L) and the newly isolated lines with respect to the expression of MHC class II molecules. Clones I and L expressed particularly low levels of class II molecules. However, when one studies the FC analysis data concerning class II expression by the newly isolated cell lines, it is clear that the expression of these molecules are substantially greater than that of clones I and L. Not only has the expression increased but the range in the amounts of class II molecules expressed by cells within the new populations has also increased. These data suggest that although the infected cells administered to the six animals were clonal, parasite transfer produced non-clonal infected cells.

Although parasite transfer has occurred and the new populations of infected cells are not clonal, CD14 expression does not differ greatly between the clones and the newly isolated lines. Both the original clones and the newly isolated lines express very little CD14, suggesting that expression of this epitope may be specifically down-regulated by the parasite. This is a particularly interesting aspect of the alterations in expression of surface molecules by macroschizont infected cells. The original line used to

produce both clones I and L was produced from cells which had been specifically selected for CD14 expression. As noted in chapter III the expression of this molecule decreases dramatically post infection/"transformation" and this is also the case when macroschizonts have transferred from a donor cell to a recipient cell.

Only cells expressing CD14 can become infected/"transformed" and produce continuously growing *T.annulata* infected cell lines (Campbell *et al*, 1994), suggesting that expression of this molecule may be important with respect to infection/infected cell development. The reasons for down regulation of CD14 post infection are not understood but appears to be of great significance as we see very low CD14 expression by cells infected with either sporozoites or macroschizonts, suggesting that the down-regulation of this molecule may be essential to aspects of infected cell behaviour.

CD14 acts as a ligand for bacterial LPS (Wright *et al*, 1990) and is a potent activator of the antimicrobial functions of MΘs (Murray and Cohn, 1980; Emmendorffer *et al*, 1990; Moncada, Palmer and Higgs, 1991). This being the case, one possible reason for the down regulation of the molecule could be to inhibit the subsequent activation of infected cells by this signalling pathway and so prevent destruction of the intracellular stage of the parasite.

Another possibility is that CD14 is down-regulated due to its link with a negative control pathway associated with the down regulation of T cell stimulation (Lue *et al*, 1991). If expression of this molecule decreases then it's activity with respect to the down-regulation of T cell proliferative responses will be reduced, allowing infected cells to alter normal T cell behaviour. However this phenomenon may simply be associated with the down regulation of MΘ activating receptors after normal cellular activation, as an attempt to control innate responses (Grey et al, 1994).

Expression of the epitope bound by IL-A 24.

In chapter III it was shown that both cells of clone I and L expressed the macrophage associated epitope bound by IL-A24. The expression of this molecule by the newly isolated cell lines generally mirrors that of the original cell lines but two lines, both produced from animals immunised with clone I express very low levels of this epitope (T.a 13274 & T.a 13254).

The expression of this molecule has been linked with the "maturity" of APCs, with Campbell *et al* (1994) suggesting that cells expressing high levels of this molecule resemble mature MΘs. Infected cells contained within lymph nodes were generally found to express higher levels an epitope bound by the mAb IL-A109 (a molecule expressed by monocytes/immature macrophages), than of the epitope bound by IL-A24 (Campbell, 1995). This may suggest that different populations of cells became infected *in vivo* than were originally infected *in vitro*, producing the original lines. But these differences may be an artefact, as placing monocytes/macrophages in culture can induce alterations in cell phenotype. Therefore *in vitro* infection may produce cells of a more mature phenotype than those infected *in vivo* and further work is necessary to determine whether this is the case.

Section 5.9.

Parasite (macroschizont) transfer.

Analysis of the cell lines isolated post immunisation showed that the parasitised cells isolated from the six immunised animals were different from the cells inoculated into the animals. This shows that parasite contained within infected cells can "cross over" to infect cells of the new host. This phenomenon has been shown before (Nichani, 1994 *inter alia*) but very little is known concerning the mechanism of parasite transfer. There are a number of different ways in which this may occur, (1) parasite transfer may be an active process, whereby macroschizonts leave infected cells and subsequently infect new cells, (2) immune reactions aimed at removing infected cells may damage parasitised cells, if the contents of the cells are released, macroschizonts may then be able to infect new cells. The most likely process, (3) is that of phagocytosis and destruction of infected cells by cells of the myeloid lineage (such as MΘs), followed by engulfed macroschizonts escaping degradation by phagocytes and establishing infections in recipient cells, an hypothesis also held by Forsyth *et al* (1997).

It is known that sporozoites are adept at gaining access to cells of the myeloid lineage (Campbell *et al*, 1994). However, there are no proven receptor/ligand pairs for the attachment and entry of sporozoites. There is also no evidence that macroschizonts express similar surface molecules to sporozoites which may aid entry into cells.

However, if one considers that during an immune reaction infected cells may be attacked and engulfed by phagocytes, this facilitates entry of parasite material into the very cells which *T.annulata* infects (Campbell *et al*, 1994; Forsyth *et al*, 1997). If this is the mechanism by which cross over occurs, after ingestion the macroschizont must escape the endosome/lysosome to prevent it's destruction. No work has been carried out in this area, we simply know that the parasite starts off in the donor cells and end up in cells of the recipient, which it subsequently "transforms". But theoretically one may imagine that the macroschizont is able either to leave the newly formed vacuoles or prevent fusion with lysosomes. Whatever happens we know that the macroschizonts are capable of infecting/"transforming" new cells and establishing an infection in recipient animals.

Characteristics of infected recipient cells & parasite transfer.

The cytokine mRNA profiles of the cell lines reisolated from the immunised animals were all essentially identical, *i.e.* all newly isolated cell lines expressed high levels of cytokine mRNAs, irrespective of the immunising cell line. Post vaccinal reactions were observed around days 13-17, the period during which the cell lines were reisolated. These newly isolated cell lines were of recipient genotype, showing that parasite "cross over" had occurred prior to reisolation. However, the severity of the reactions correlated with the cytokine profile of the immunising cell line and not with that of the newly isolated line.

Given that post vaccinal reactions appear to be related to the presence of a high cytokine producing/T cell stimulating cell line (clone L) and are not related to the "pathogenicity" of cells infected following parasite transfer, the following mechanism for the induction of post vaccinal reactions may be proposed (Diagram. 5.1). Infected cells administered to animals will continue to proliferate *in vivo* until cleared by the immune system. Nichani (1994) showed that CTL responses against immunising cell lines peaked at 11 to 12 days post immunisation and persisted until day 17/18. It is therefore likely that the largest numbers of immunising cells are present during the first part of this time period, coinciding with the peak of post vaccinal reactions. Post vaccinal reactions decline from day 16 to 17 coinciding with the reduction in the CTL response to the immunising MHC and presumably the immunising cell line.

Diagram 5.1

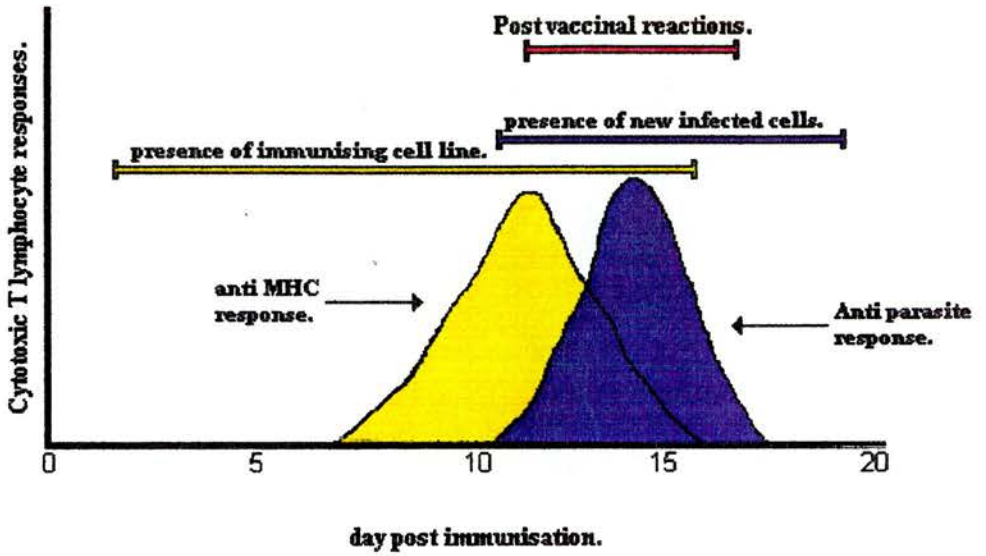


Diagram. 5.1 **A possible time scale of responses to macroschizont infected cell lines when used to immunise animals against *T.annulata* infection.**

The time scale of host CTL responses against (1) the immunising cell line (anti-MHC response), followed by (2) the response to parasitised cells of the recipient (anti-parasite response) (Nichani, 1994). Post vaccinal reactions to clone L appear to coincide with the peak of the anti-MHC response and wane as cells of the immunising cell lines are destroyed.

Conclusion.

The data presented within this chapter shows that within the bounds of these experiments, the two different cell lines (I and L, derived from the same parent line), produce different levels of pathology when administered to animals but that both induce protective immunity in these otherwise susceptible animals. Therefore, it is possible using the techniques of soft agar cloning, RT-PCR analysis and T cell proliferation studies to produce and assess the suitability of new *T.annulata* macroschizont infected vaccine cell lines over a much shorter time scale than can generally be accomplished. If these techniques can be developed further and more information concerning the factors governing attenuation collected, then their implementation in the area of vaccine production may greatly increase the efficiency of vaccination programs throughout the developing world.

Chapter VI.

Conclusions and Future work.

Summary and conclusions.

Infection of cattle with the intracellular protozoan parasite *T.annulata* causes severe illness, especially in exotic breeds (Robinson, 1982). This organism infects cells of the immune system, namely monocytes and MΘs (Glass *et al*, 1989; Spooner *et al*, 1989; Campbell *et al*, 1994), transforming the cells it infects and inducing alterations in infected cell behaviour. Soon after transformation infected cells possess the ability to induce the activation and proliferation of autologous T cells. Possibly the most important effect that *T.annulata* infected cells have upon T cells is to drive the T cell response towards a non-protective Th_1 type response (Campbell *et al*, 1997). The induction of this type of response in susceptible cattle is followed by destruction of the architecture of the draining lymph node, production of high levels of cytokines (such as IFNγ) and ultimately the failure of the host to mount a protective response against the parasite (Campbell, 1995; Campbell *et al*, 1997). To fully understand and therefore develop protective strategies against this parasite one must decern the exact mechanisms by which the infected cells alter T cell and immune function.

This thesis concentrates upon the effects which *T.annulata* has upon the cells which it infects (moncytes and MΘs) and details research into aspects of tropical theileriosis, including the immunological functions of infected cells (T cell stimulatory ability and cytokine production) and possible causes of the immunopathology observed post infection. Protection of susceptible animals is possible by vaccination with long term tissue culture attenuated cell line vaccines protects animals from infection in the field. However, animals can suffer post vaccinal reactions, causing morbidity and reducing productivity. Knowledge of the exact causes of the pathology associated with *T.annulata* infection and post vaccinal reactions remains incomplete. A better understanding of the mechanisms which induce pathology would allow the production of more efficient vaccines, reducing the levels of morbidity post immunisation.

T.annulata infected monocytes and MΘs possess enhanced antigen presentation function and are also capable of inducing high levels of T cell proliferation (Glass & Spooner, 1990; Campbell *et al*, 1995). The presence of inflammatory cytokines during *T.annulata* infections has also been linked with the exacerbation of disease (Preston *et al*, 1992b). Glass and Spooner (1990) also demonstrated that APCs generally express elevated levels of MHC class II antigens following *T.annulata* infection. They postulated that the increased levels of MHC class II expression could account for the enhanced antigen presenting function of infected cells and may be related to the alteration of T cell function by infected cells.

During the course of this thesis Campbell *et al* (1995) highlighted possible mechanisms involved in the immunopathology of this disease. Inappropriate T cell activation and the contact specific induction of non-specific T cell proliferation by infected cells produces a non-protective Th_1 helper like response (Campbell *et al*, 1997). This T helper response is accompanied by the presence of extremely high levels of IFNγ in the periphery (20x the levels observed in a protective immune response) (Campbell *et al*, 1997).

With the identification of the mechanisms which may underlie pathogenesis, an understanding of the factors driving these reactions became essential. The aims of the thesis were therefore to (1) quantitate the expression of MHC class II molecules by infected cells, (2) to relate this to T cell stimulatory ability and (3) investigate other factors which may influence the stimulation of T cells by macroschizont infected cells.

Infected cell MHC class II expression & T cell stimulatory ability.

Clonal populations of *T.annulata* infected cells were produced from a defined population of $CD14^+$ cells, by the novel use of soft agar cloning. This allowed the quantitation of MHC class II expression by specific populations of infected cells and investigation of links between class II expression and T cell stimulatory ability. MHC class II expression varied between the four infected cell populations studied. The uncloned parental line expressed the highest levels of MHC class II per cell, whilst

of the clonal lines clone G expressed higher levels of MHC class II than clones I or L (which expressed similar levels of these molecules). Quantitation of MHC class II expression of these cultures also showed that there is little fluctuation in class II expression by *T.annulata* infected cells over time in culture.

When these four different infected cell populations were used to stimulate autologous PBM it was found that the levels of T cell proliferation induced did not correlate with the observed levels of MHC class II expression. The line which reproducibly induced the highest levels of proliferation was clone L. This line expressed similar levels of MHC class II molecules to clone I. However, clone I induced much lower levels of T cell proliferation. Clone G which expressed higher levels of class II than both clones I and L induced levels of T cell proliferation between those induced by clones I and L and the parent line which expressed the highest levels of class II induced similar amounts of T cell proliferation to clone G. These results therefore show that the levels of MHC class II expressed by *T.annulata* infected cells do not correlate to the levels of T cell proliferation induced, meaning that enhanced expression of these molecules can not be the only requirement for the induction of T cell proliferation.

A further point which should be noted is that all three clonal populations studied expressed lower levels of MHC class II molecules than the original population of infected cells. This may suggest that soft agar cloning selects for infected cell populations which express low levels of these molecules. A further ten clonal populations were produced by cloning of the parent line which were not studied due to lack of time. However it would be interesting to know whether soft agar cloning does impose selection pressures, which result in the isolation of infected cell populations of similar phenotype (with respect to MHC class II expression).

There are a number of possible reasons for the differences in T cell proliferation induced by these infected cell populations. When one considers the MHC class II expression of the clonal cultures it is apparent that although they do possess narrower ranges of MHC class II expression than the uncloned line (T.a 12929), variation does remain within the clonal populations. Therefore one wonders if particular subpopulations (which express different levels of MHC class II molecules) within clones G, I and L, vary in the way in which they affect autologous T cells. The data

in this thesis relating to the connection of MHC class II expression to T cell proliferation does not address this question, as MHC class II expression was determined as the average for the whole populations and does not shed any light upon the ways in which inter-population variation in MHC class II expression may affect T cell proliferation. This possibility could be addressed if the clonal lines were subcloned by FACS sorting on the basis of MHC class II expression, followed by investigation of the proliferative response of T cells to the subclones expressing different levels of MHC class II.

Variation in MHC class II expression may also be produced as different cells within the population move through the cell cycle. Attempts to minimise variation which may result due to this phenomenon were made and involved the harvesting of infected cell populations only whilst in the log phase of growth (12-18 hours after passage). However, this possibility could be investigated further by using flow cytometric analysis to assess the stage of the cell cycle which infected cells occupy and the levels of MHC class II they express.

Another reason for the variation in MHC class II expression may be that although the clonal cultures were derived from single infected cells variation in MHC class expression may arise due to the presence of more than one parasite within the infected cells. This would mean that although the cultures are clonal with respect to the cells, they may not be clonal with respect to the parasite they contain. To remove this variation and allow further investigation into the relationships between MHC class II expressed by infected cells and autologous T cell proliferation one would need to subclone cultures G, I and L, in an attempt to obtain infected cell lines with even narrower expressions of MHC class II. Genetic analysis could also be carried out to ascertain whether the clonal lines did contain more than one parasite per population. If subcloning of the populations proved unsuccessful one would need to produce new clonal lines which were produced from a single cell and a single sporozoite.

It must also be remembered that there are many other cell surface molecules apart from MHC class II molecules, which play roles in T cell activation (*i.e.* B7 and ICAM1). At the time that these investigations took place there were very few mAbs available which bound specifically to bovine cell surface molecules involved in T cell

activation. As a result the expression of T cell stimulatory accessory molecules by the clonal lines were not investigated. It is therefore possible that the levels of accessory molecules expressed by infected cell lines (along with cytokines produced by infected cells (see below)) play a part in determining the levels of T cell proliferation induced. Before one can decisively show which molecule or combination of molecules is necessary to induce various levels of autologous T cell proliferation one must investigate the expression of other important T cell stimulatory molecules by these cell lines.

A further point of interest concerns the phenotype of the cells, which the infected cell lines used during this study were produced from. Spooner *et al* (1989), Glass *et al* (1989) and Campbell *et al* (1989) had shown that the target for *T.annulata* sporozoites were monocytes and MΘs. The infected cell lines produced for this study were therefore produced from purified populations of $CD14^+$ monocytes. The cells transformed by infection with this parasite were $CD14^+$ prior to infection but rapidly became $CD14^-$ after the establishment of an infected cell population. These findings have since been confirmed by Sager *et al* (1997) who showed that infection/transformation of cells by *T.annulata* induced a down regulation of CD14 expression at the level of the gene. This is an example of a parasite interfering with transcriptional events and a similar mechanism may be employed by the parasite to alter the levels of MHC class II expressed by infected cells. The actual mechanisms by which the parasite brings about these changes remain unknown and much research is therefore necessary to elucidate the factors produced by *T.annulata* which are capable of altering mammalian gene expression. However, determination of the mechanisms which *T.annulata* employs to alter gene expression may prove valuable in the investigation of the ways in which other intracellular protozoan parasites affect mammalian cells.

Cytokine mRNA production by Theileria infected cells.

RT-PCR analysis of the production of a range of T cell stimulatory and proinflammatory cytokines by the four *T.annulata* infected lines showed that the presence of this parasite within cells induces the constitutive production of cytokine mRNAs specific for IL-1α, IL-1β, IL-6, IL-10 and TNFα. All of the four *T.annulata* infected lines produced similar cytokine mRNA profiles, however, a cell line infected with the related parasite *T.parva* produced a different profile of cytokine mRNAs (including, IL-1α, IL-2, IL-4, IL-10 and IFNγ). The finding that cells infected with either *T.annulata* or *T.parva* produce mRNA specific for IL-1α suggests that this may be an important part of the mechanisms involved in T cell activation by infected cells.

Limiting cycle RT-PCR analysis allowed comparison of the levels of cytokine mRNAs produced by the different populations of infected cells. Clone I produced the lowest levels of the majority of the cytokine mRNAs, whilst clone L expressed the highest and clone G and the parent line made these cytokine mRNAs in roughly similar amounts. These findings correlate with the levels of T cell proliferation observed after incubation of autologous T cells with infected cells (*i.e.* clone L produces higher levels of T cell stimulatory cytokines than clone I and also produces substantially more T cell proliferation). A direct correlation was shown between the levels of cytokine mRNAs produced by infected cells (with particular emphasis on production of IL-1α and IL-6 mRNAs) and the levels of T cell proliferation observed. This suggests that although the T cell activation induced by macroschizont infected cells is contact specific (Campbell *et al*, 1995) the T cell proliferation maybe driven by cytokines.

However, one must remember that the analysis carried out on these infected cell lines only investigates the presence of mRNA species specific for various cytokines and not functional proteins. It is therefore possible that even though T cell proliferation levels do correlate with the expression of cytokine mRNAs specific for IL-1α and IL-6, some or all of these cytokines may be expressed only at the mRNA level. To obtain a clearer picture of the causes of *T.annulata* induced T cell proliferation one needs to

use bovine specific bioassays to ascertain the functional expression of the cytokines in question.

Recent findings by Sager *et al* (1997) show that *T.annulata* infected cells are capable of producing both IL-1 and TNFα but that the TNFα produced is not secreted from the infected cells *in vitro*. Biologically active TNFα was only found within cell lysates and may suggest that this cytokine is present only in an intracellular or membrane bound form, possibly playing a role as a autocrine signal. Another possibility is that TNFα is produced and released *in vivo*, possibly when infected cells interact with other cells of the immune system. However, IL-1 activity was found and this may have relevance, as IL-1 is capable of inducing both pyrexia and cachexia via its effects upon other cells of the immune system. This confirmation that *T.annulata* infected cells are capable of producing biologically active proinflammatory and cachexia inducing cytokines strengthens the evidence for a link between the production of such cytokines by infected cells and the induction of pathology and disease within *T.annulata* infected animals.

Another facet of tropical theileriosis is the induction of the production of IFNγ by T cells stimulated with macroschizont infected cells, shown both in this thesis at the mRNA level (also Campbell *et al*, 1995) and at the protein level (Campbell *et al*, 1997). This cytokine generally acts in a protective way during protozoan infections (*i.e. T. gondii* and *Leishmania. spp.* infections). However, the non protective Th_1 type response and high levels of IFNγ induced by the presence of *T.annulata* infected cells appears to exacerbate the infection not clear it, possibly by inducing large scale T cell activation and disruption of the immune response (Campbell *et al*, 1997). This thesis therefore shows *T.annulata* to be a novel parasite which does not conform to the established rules concerning the immunology and immunopathology of the majority of intracellular protozoan parasites.

Use of clones I & L to immunise naive susceptible animals.

In vitro characterisation showed clones I and L possessed markedly different abilities to stimulate autologous T cells and produced different amounts of T cell stimulatory and proinflammatory cytokine mRNA species. These characteristics are thought to be of great importance in the production of pathology and the inhibition of a protective immune response during a *T.annulata* infection (Campbell, 1995; Campbell *et al*, 1997). There is also a possibility that these characteristics are responsible for the production of the post vaccinal reactions sometimes observed after vaccine administration, which can include fever, anemia, lethargy and loss of appetite (Pipano, 1981).

When animals were immunised with cells of these two clonal cultures the reactions observed post immunisation correlated with the *in vitro* characteristics of the immunising culture. Clone L which produced the highest levels of T cell proliferation and stimulatory cytokines also induced signs of greater severity than clone I, including greater rises in temperature and greater falls in PCV and leucocyte numbers. The Gharb sporozoites used to challenge these animals produced severe signs in both control animals (also when used in experiments by Dr Anil Nichani (1994)). However, all six cattle (irrespective of the immunising cell line) were protected by vaccination with these low passage cell lines.

The major difference between the selection procedure I employed and the traditional methods of cell line selection is that of soft agar cloning. This method does not rely upon the long term culture of infected cells to produce attenuated clonal populations of parasite and cells. It selects single infected cells containing unattenuated (low passage) parasite which can then be grown on to form populations which can be screened for an attenuated phenotype.

The long term attenuation of cell line vaccines can lead to their over attenuation, producing reduced levels of protection (Dargouth *et al*, 1996; Ilhan *et al*, 1997). The rapid production of cell lines using this new method circumvents these problems, allowing the preparation of vaccine lines from cells with an attenuated phenotype which contain unattenuated/immunogenic parasite material. The importance of cellular

phenotype, with respect to the virulence of infected cell lines is indicated by the results obtained after RT-PCR analysis of cytokine mRNA production of the newly isolated cell lines. These cell lines were produced by *in vivo* parasite "cross over", during which non clonal populations of cells became infected. All of the newly isolated cell lines assessed produced high levels of T cell stimulatory/proinflammatory cytokines. This suggests that the virulence of infected cells depends not only upon the parasite they contain but also upon the cells which become infected. Therefore selection of future vaccine lines on the basis of cellular phenotype and not necessarily parasite alone, may dramatically increase the efficiency of vaccine production/selection.

I have shown that the *in vitro* T cell proliferation induced correlates with the levels of T cell stimulatory cytokine mRNA produced by macroschizont infected cells. It also appears that the levels of post vaccinal reactions induced also correlate with the levels of cytokine mRNA produced by the immunising cell line. I therefore feel that this validates the use of limiting cycle RT-PCR to aid in predicting the virulence of putative cell line vaccines and shows that the criteria upon which these two immunising cell lines were chosen may be suitable for the selection of vaccines against tropical theileriosis. Also that this technique could possibly be used to screen putative vaccine lines in a fraction of the time normally required.

However, before this method can be used on a practical basis more detailed investigations must be carried out into this techniques ability to predict the virulence of new cell line vaccines. It must be remembered that this was a small scale study and the differences detected between the reactions of the two groups of animals to the two cell lines, although statistically significant for three parameters (temperature, PCV and TLC) were not particularly different and none of the six animals required treatment. Also if one studies the data relating to the changes in PCV and total RBC numbers one sees that the PCV falls (indicating loss of RBCs and approaching anemia) but the actual numbers of RBCs do not fall as might be expected. Therefore further study of the affects of immunisation with these two cell lines should be carried out using a larger number of animals, with particular detail payed to variations in blood composition. Base line measurements for all the parameters should be taken for around

six to eight days prior to immunisation and the numbers of animals in each group should be increased, to ease statistical analysis of resulting data. Differential cell counts should also be performed on daily blood samples during the experiment. This would allow fluctuations in the numbers of different cell types (*i.e.* lymphocytes, neutrophils and eosinophiles) in the peripheral circulation to be calculated while the animals respond to the presence of the theileria parasite.

One factor which I would have liked to study would have been the response of animals to the cell lines derived from clone I, which were isolated post parasite transfer and found to express higher levels of cytokine mRNA than the original clone I. If the hypothesis that cytokine production by the immunising cell line plays an important role in the induction of pathology and the determination of the course of infection proved correct, one may expect to observe that animals immunised with the cell lines produced following parasite transfer (which possessed cytokine mRNA profiles resembling that of clone L) would suffer more severe signs than those induced by immunisation with the original clone I. This would suggest that the phenotype of the cells infected and not simply the phenotype of the parasite is important with respect to attenuation and also in determining the behaviour of the cells after infection and therefore the severity of disease which they are capable of inducing. This differs from the traditional ideas of why cell lines may be virulent or avirulent, as virulence is normally attributed to the characteristics of the parasite alone.

Further investigation into the relationships between the cytokine production of infected cell populations and the induction of immunity versus pathology is necessary before limiting cycle RT-PCR analysis can be used to safely assess cell line vaccines. However, the data in this thesis suggests that this method of analysis of macroschizont infected lines, if found reliable, would prove a much cheaper and quicker method of characterising putative vaccines for use against tropical theileriosis.

Future work.

Much investigation into the immunopathology of tropical theileriosis still remains to be done before we have a true picture as to the exact mechanisms which the parasite employs to interfere with the host immune system and induce pathology. However, the evidence to date suggests that cytokines including, IL-1, IL-6 and TNFα, derived from infected cells play a crucial role in the induction of both the inappropriate T cell activation and pathology (including fever, anemia and cachexia) observed following infection. However, before we are able to fully understand the roles of each cytokine produced by infected cells in the induction of *in vivo* responses to *T.annulata* infection, experiments must be conducted to delineate the reactions observed post infection/immunisation.

Two possible routes which would allow the dissection of the highly complicated interactions involved in *T.annulata* infection are (1) the development of bovine specific cytokine bioassays and (2) the production and characterisation (both *in vitro* and *in vivo*) of more early passage clonal cell lines. Assessment of the production of biologically active cytokines is necessary before one can be totally certain of the production/secretion of active cytokine molecules by infected cells. This could also aid the development of blocking assays to determine the specific roles of individual or combinations of cytokines, with respect to non-specific autologous T cell activation. To assess the specific roles of cytokines with respect to the induction of pathology, the production and characterisation of a range of clonal lines, expressing different ranges of cytokines and the immunisation of cattle with these lines, would facilitate study of which cytokines/combination of cytokines are important in altering *in vivo* immune responses and inducing pathology. If these studies were to prove successful they would not only greatly improve our knowledge of this parasite but could also allow *T.annulata* vaccine production to be carried out using a sound scientific basis and dramatically increase it's efficiency.

Limiting cycle RT-PCR analysis could also be used to study the production of cytokine mRNAs by established vaccine lines and would show whether lines which are routinely used to immunise animals against *T.annulata* also produce low levels of inflammatory cytokines. If these cell lines do produce low levels of these molecules it would be further evidence to suggest that cytokine production may be linked with the induction of pathology. However, if these cell lines do not produce low levels of inflammatory cytokines then it may suggest that the attenuation of the parasite plays a bigger role in the induction of immunity and pathology, than the phenotype of the infected cells. Also that the attenuation of early passage lines (as is seen in this thesis) may occur by a different mechanism than in lines produced by traditional long term tissue culture methods. Cytokine production may not be as an important a characteristic in cell lines where the parasite has been attenuated over long periods in culture. However, much more information is needed before possible differences in the mechanisms of attenuation can be identified. However, isolation and study of infected cells from animals following immunisation with conventionally produced vaccine lines, may reveal if cytokine mRNA expression generally increases after parasite cross over from donor to recipient cells (as in the case for clone I) and whether this does relate to the state of attenuation of infected cells.

This technique of assessing the production of cytokine mRNAs could also be utilised in other systems where infected/parasitised cells induce pathology. Allowing analysis to be carried out upon animals of the host species and possibly removing some of the reliance on model systems. *T. gondii* and *Leishmania. Spp*, both infect macrophages and although they do not "transform" the cells infected they are able to induce changes in the expression of cytokines by the infected cells (Khan *et al*, 1996; Chakkalath and Titus, 1994). This method of limiting cycle analysis could prove useful in investigating cytokine production by infected cells and delineating the immune reactions induced by the presence of other parasites. One example of this concerns the investigation of the ruminant intra-macrophage parasite *Cowdria ruminatum* (Allsopp *et al*, 1997). This parasite causes the disease heartwater and affects many animals in the third world. The presence of infected cells appears to induce the production of a Th_2 response (Dr K. Sumption, pers. comm.), a

characteristic they share with *Leishmania* infected cells (Chakkalath and Titus, 1994) Limiting cycle analysis may allow any involvement of cytokine production by infected cells within the bovine immune system to be investigated. This is one example where use of this technique could help delineate immune reactions within the host, without the need to use model infections in mice or rats, which may produce data not pertinent to the real infection.

As stated *T.annulata* infected cells possess the ability to alter the behaviour of T cells which they come into contact with (Campbell, 1995; Campbell *et al*, 1997). This is not unique amongst protozoan parasites, as others including, *Plasmodium yoelii* (Pied *et al*, 1997), *Trypanosoma cruzi* (Gomes *et al*, 1996), *Leishmania major* (Zimmermann *et al*, 1994) and *Toxoplasma gondii* (Khan *et al*, 1996) have all been shown to possess the ability to alter T cell responses. Two of these examples show parasites able to induce T cell unresponsiveness (Gomes *et al*, 1996; Khan *et al*, 1996). However, there is evidence to suggest that *L. major* and *P. yoelii* possess superantigen like activities. *L.major* has been found to share an epitope with the murine CD3-T cell receptor complex and the ability to activate T cells (Zimmermann et al, 1994). Whilst both sporozoites and erythrocytic stages of *P. yoelii* have the ability to induce large scale T cell activation and the deletion of Vβ9 T cells (Pied *et al*, 1997). *T.annulata* infected cells share this ability but whether T cell activation occurs via a similar mechanism remains to be seen.

The identity of the molecule expressed on the surface of *T.annulata* macroschizont infected cells, which is responsible for the contact specific T cell activation signal and the roles played by particular cytokines during this process remains unknown. However, further study of this and other parasites may provide clues as to the way in which *T.annulata* is able to activate such a large proportion of the circulating T cell pool during an infection (Nichani, 1995). Therefore, further studies should investigate the expression of a wide range of surface molecules by infected cells, such as the B7-1 and B7-2 molecules, to assess those which may play a part in the contact specific activation of autologous T cells. Also the expression of biologically active cytokines and the specific roles which they play in *T.annulata* induced T cell activation should be investigated.

Although there are some similarities between certain characteristics of *T.annulata* and those of other protozoan parasites, *T.annulata* is the only one known which is able to cause the dedifferentiation and "transformation" of infected monocytes and macrophages (Campbell *et al*, 1994; Sager *et al*, 1997), whilst also inducing septic shock like immunopathology, severe enough to kill the animal within twelve to twenty days post infection (depending on parasite dose). Therefore, it is I feel very important for all the molecules involved in the production of the non protective Th_1 type immune response to be elucidated before we can obtain a clear picture of how this protozoan parasite disrupts the immune system and produces the severe signs associated with tropical theileriosis. This knowledge could hopefully then be used to produce a molecular vaccine which could induce the immune system to form a protective response to *T.annulata* infection. Thus the need for the costly production and use of potentially dangerous live cell line vaccines could be eliminated.

Chapter VII.

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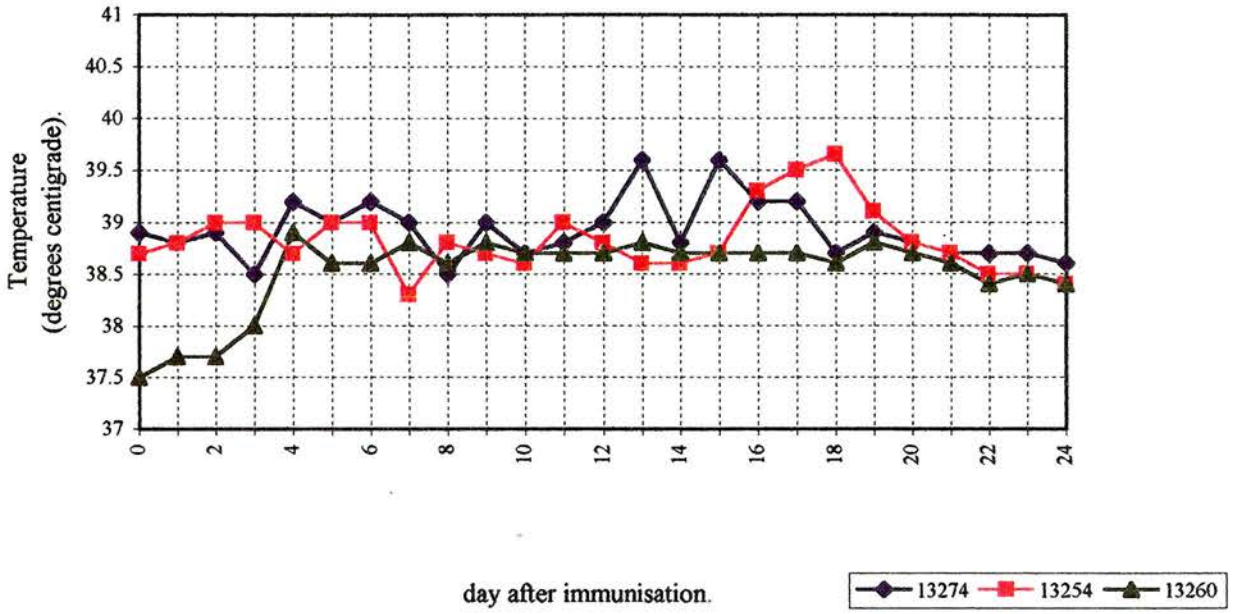
Appendix.

App 5.1 **Temperature data from animals 13274, 13254 and 13260 after immunisation with cells of clone I.**

App 5.2 **Temperature data from animals 13266, 13271 and 13249 after immunisation with cells of clone L.**

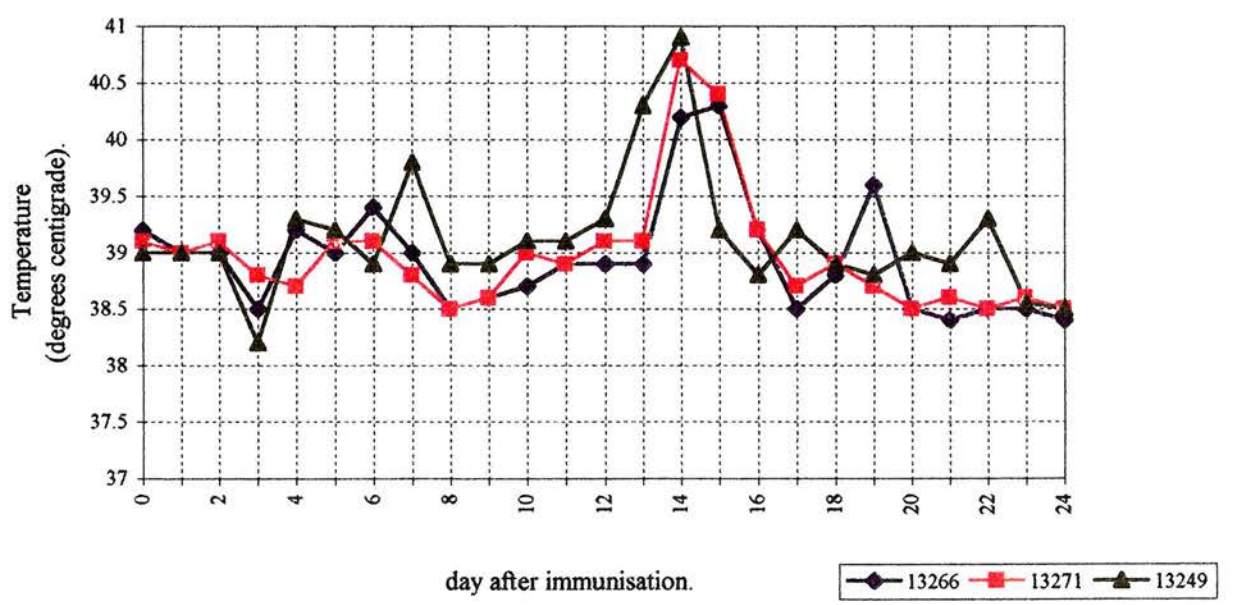
App 5.1.

Temperatures of animals immunised with clone I.



App 5.2.

Temperatures of animals immunised with clone L.

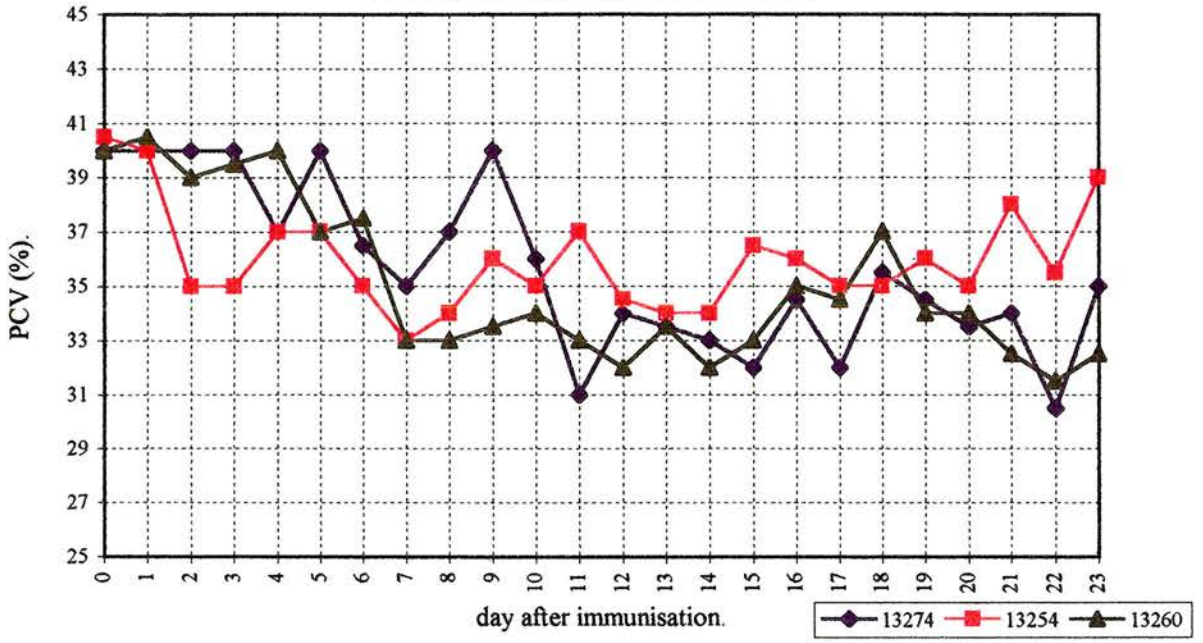


App 5.3 **PCV data from animals 13274, 13254 and 13260 after immunisation with cells of clone I.**

App 5.4 **PCV data from animals 13266, 13271 and 13249 after immunisation with cells of clone L.**

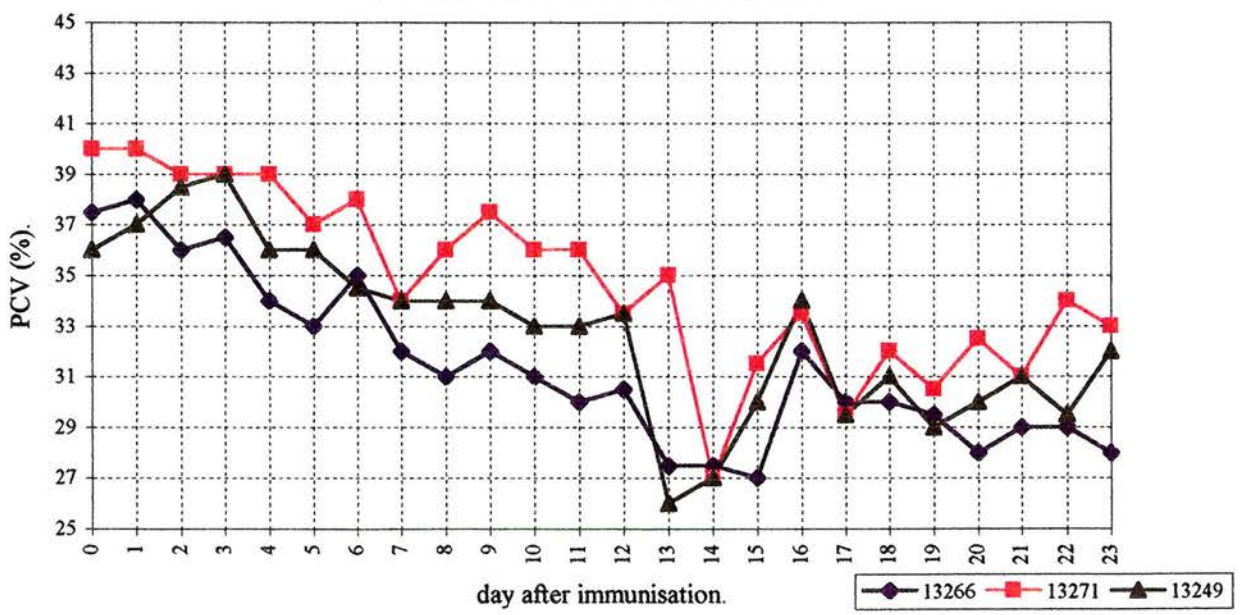
App 5.3.

PCVs of animals immunised with clone I.



App 5.4

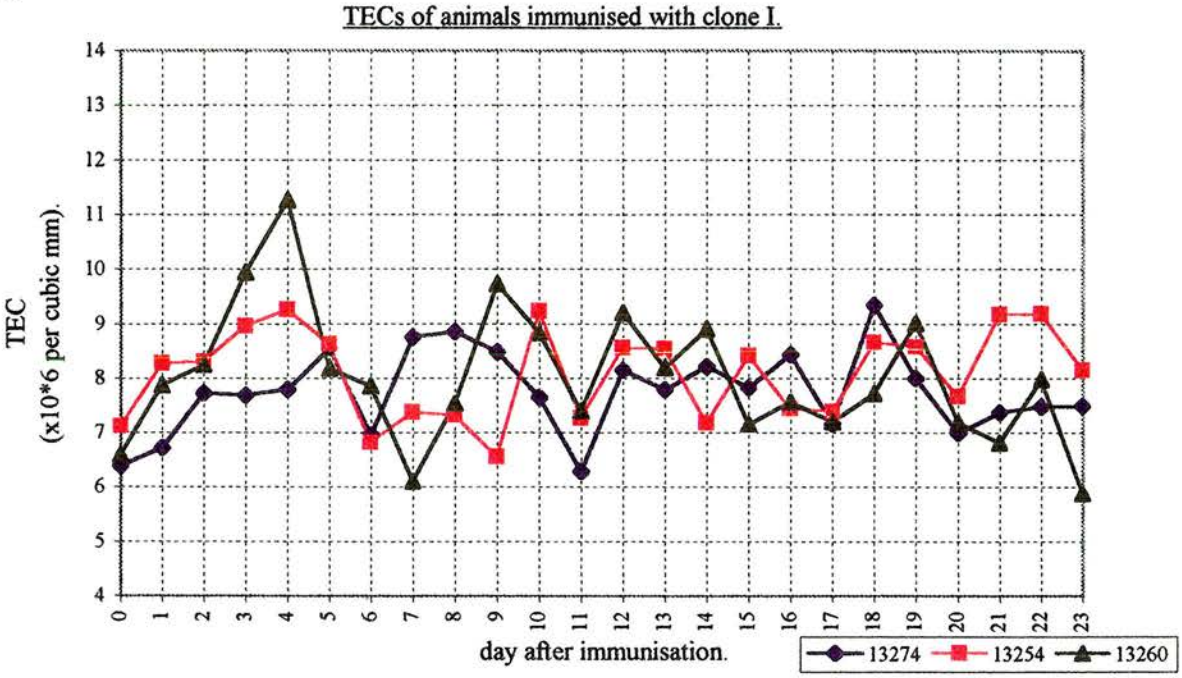
PCVs of animals immunised with clone L.



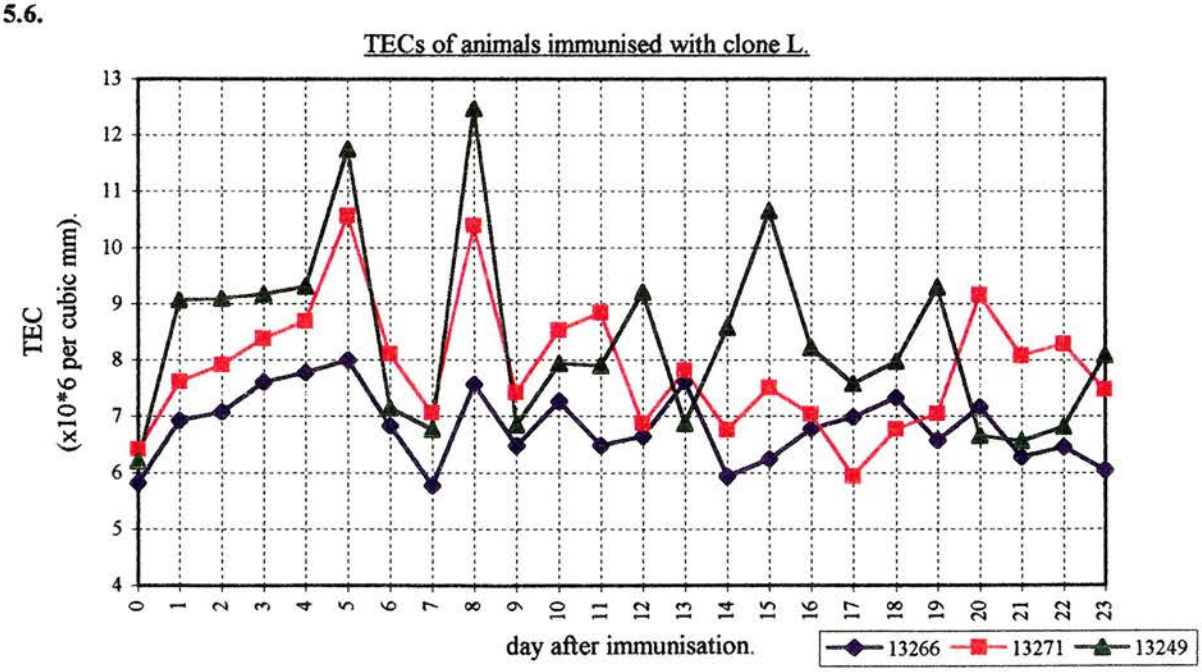
App 5.5 **TEC data from animals 13274, 13254 and 13260 after immunisation with cells of clone I.**

App 5.6 **TEC data from animals 13266, 13271 and 13249 after immunisation with cells of clone L.**

App 5.5.


TECs of animals immunised with clone I.
TEC
(x10*6 per cubic mm).
day after immunisation.
13274
13254
13260

App 5.6.

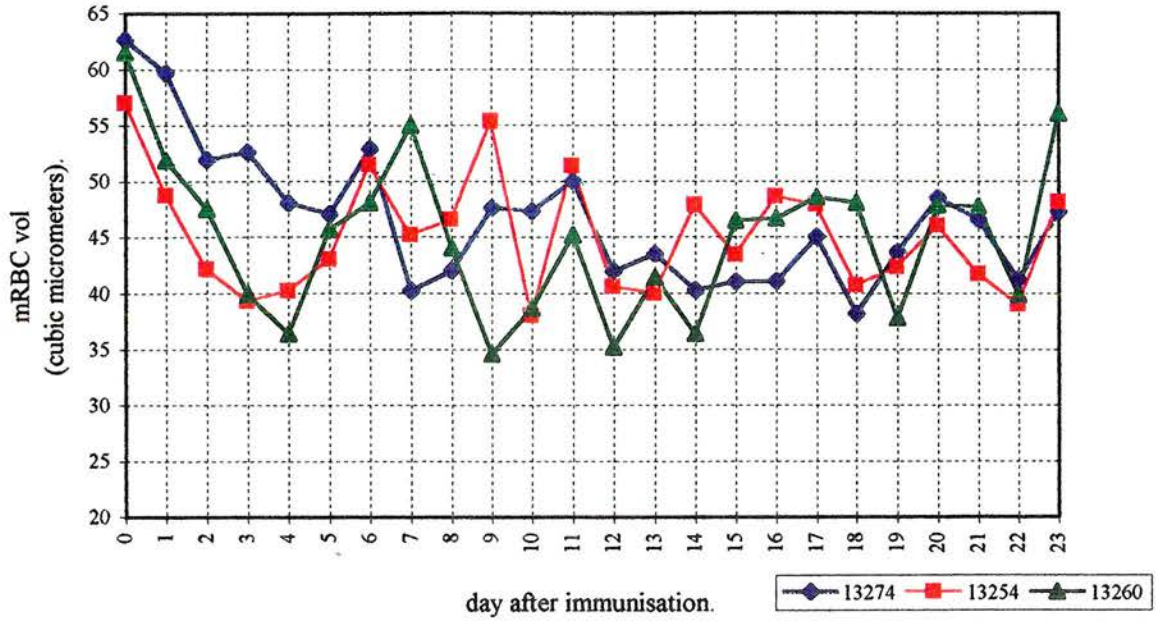

TECs of animals immunised with clone L.
TEC
(x10*6 per cubic mm).
day after immunisation.
13266
13271
13249

App 5.7 **Mean RBC volume data from animals 13274, 13254 and 13260 after immunisation with clone I.**

App 5.8 **Mean RBC volume data from animals 13266, 13271 and 13249 after immunisation with clone L.**

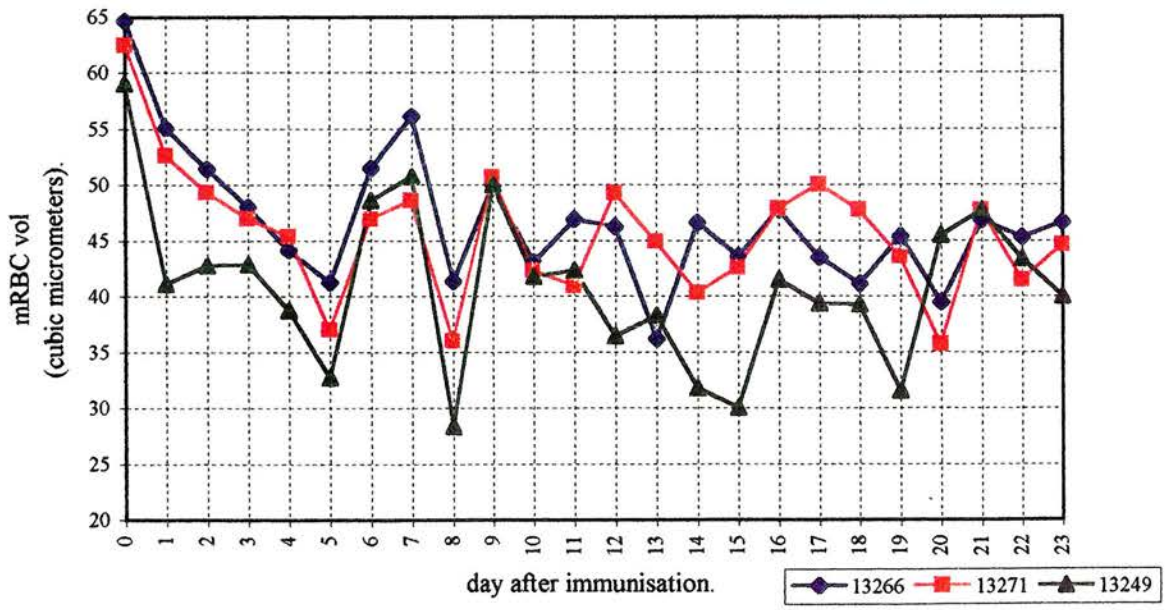
App 5.7.

Mean RBC volume of animals immunised with clone I.



App 5.8.

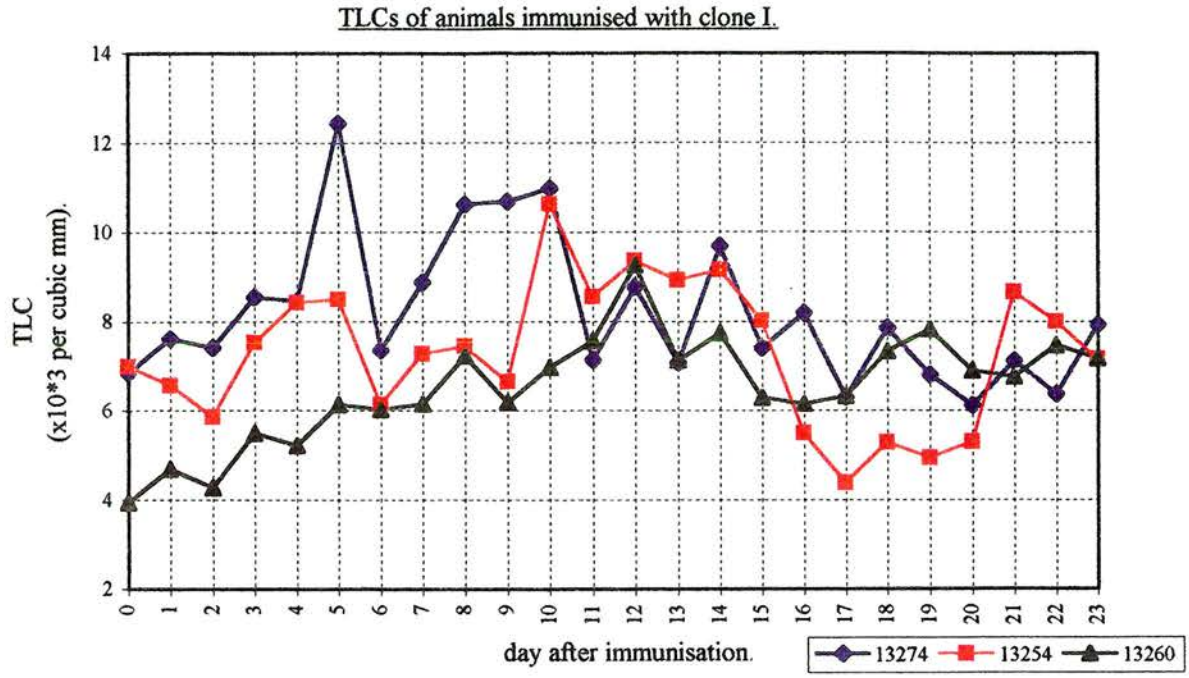
Mean RBC volumes of animals immunised with clone L.



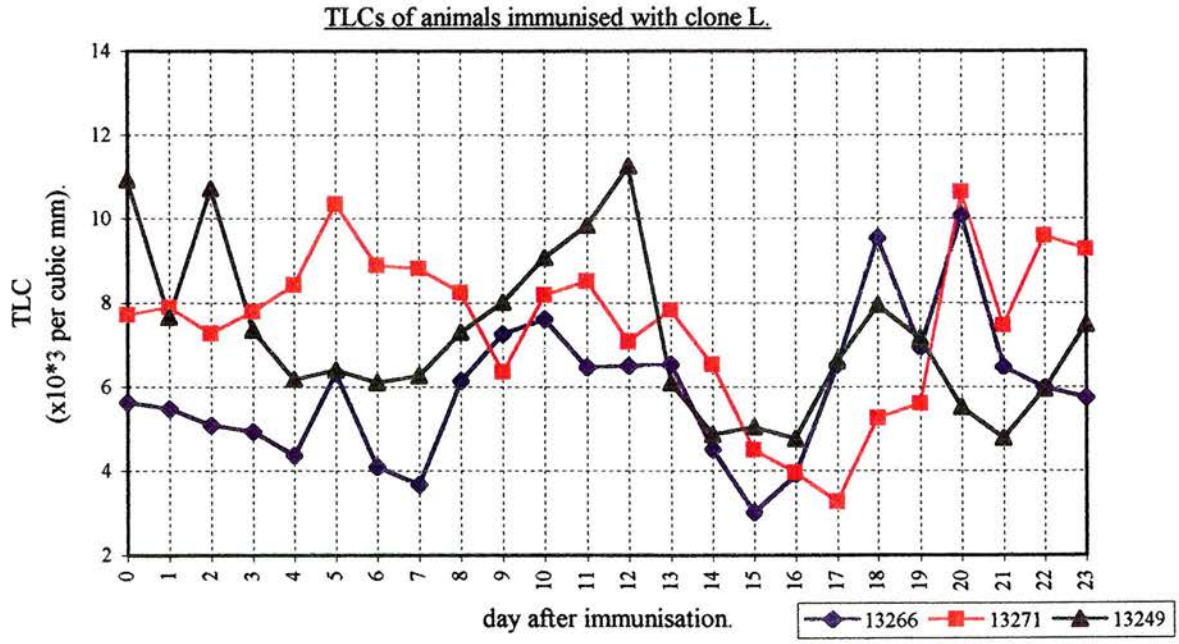
App 5.9 **TLC data from animals 13274, 13254 and 13260 after immunisation with clone I.**

App 5.1.0 **TLC data from animals 13266, 13271 and 13249 after immunisation with clone L.**

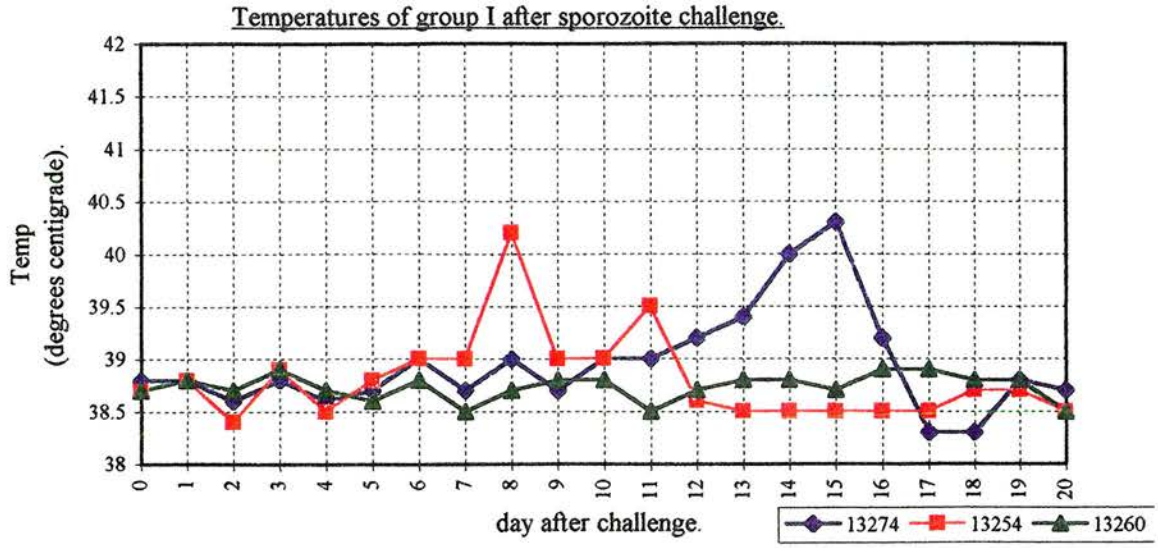
App 5.9.



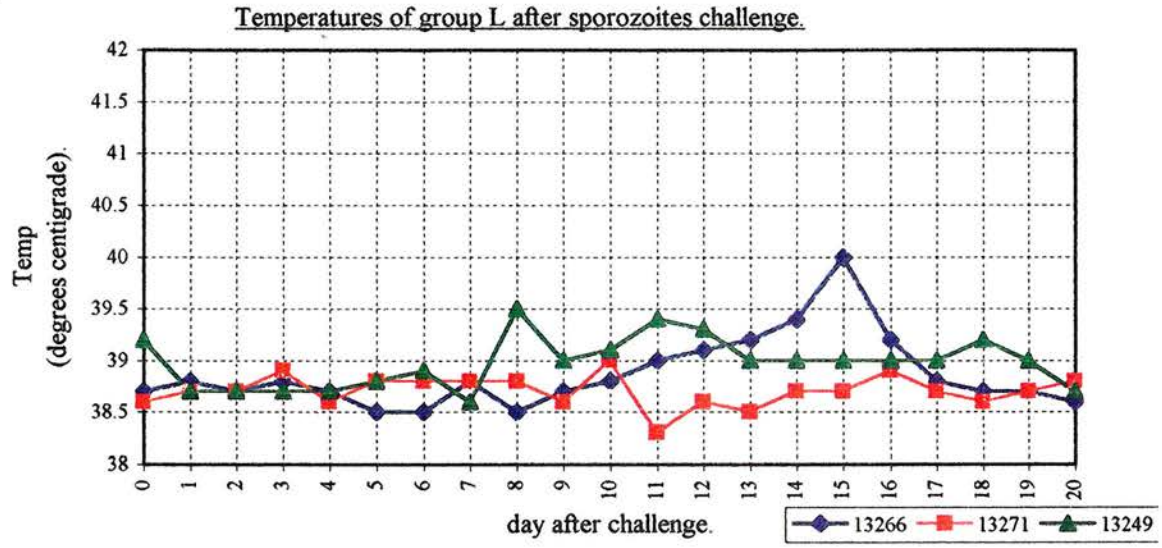
App 5.1.0.



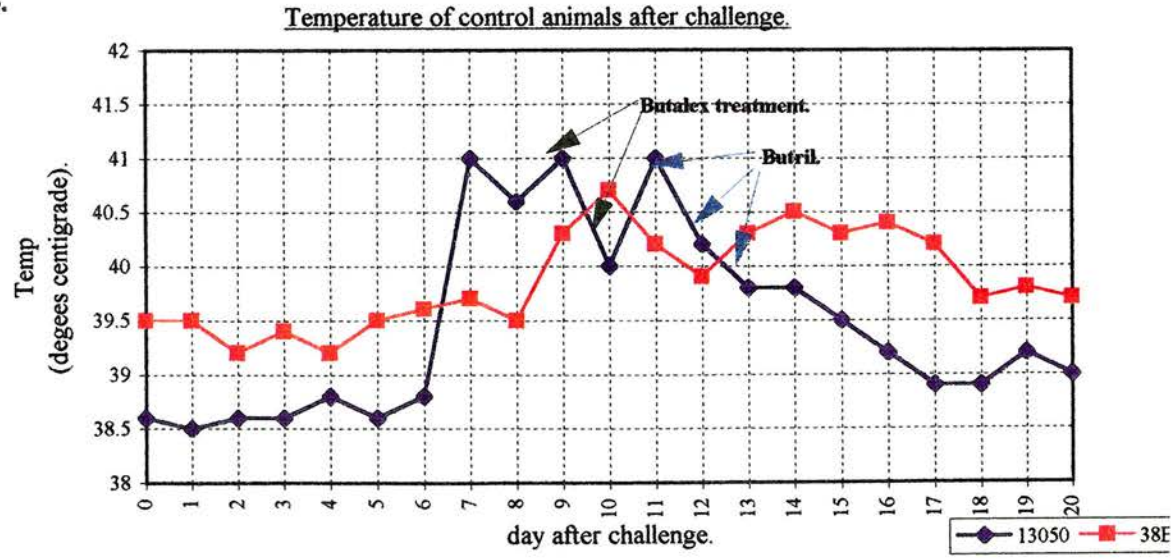
App 5.1.1.



App 5.1.2.



App 5.1.3.



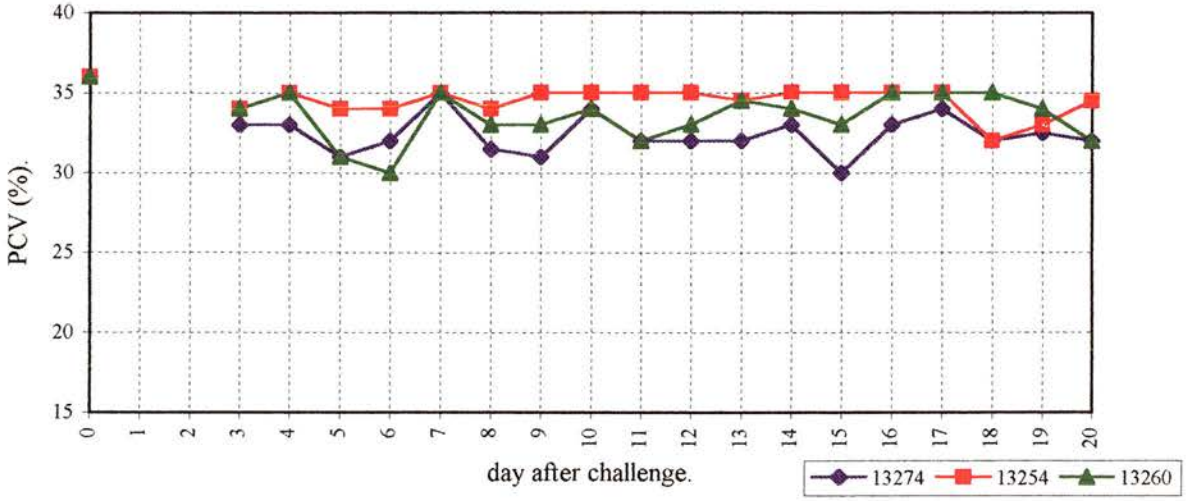
App 5.1.4. PCV data from animals from group I post challenge.

App 5.1.5. PCV data from animals from group L post challenge.

App 5.1.6. PCV data from control animals post challenge.

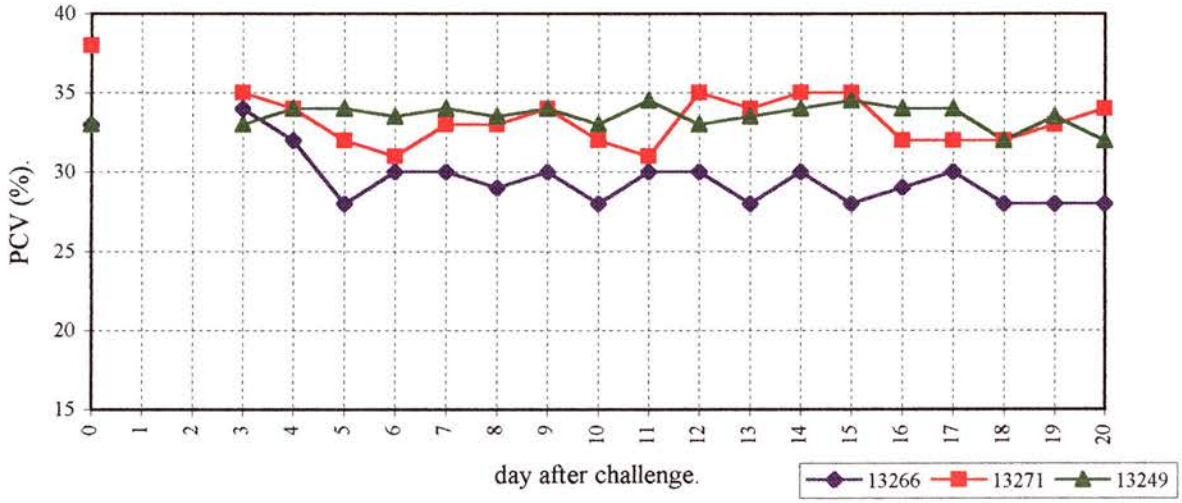
App 5.1.4.

PCVs of group I after sporozoite challenge.



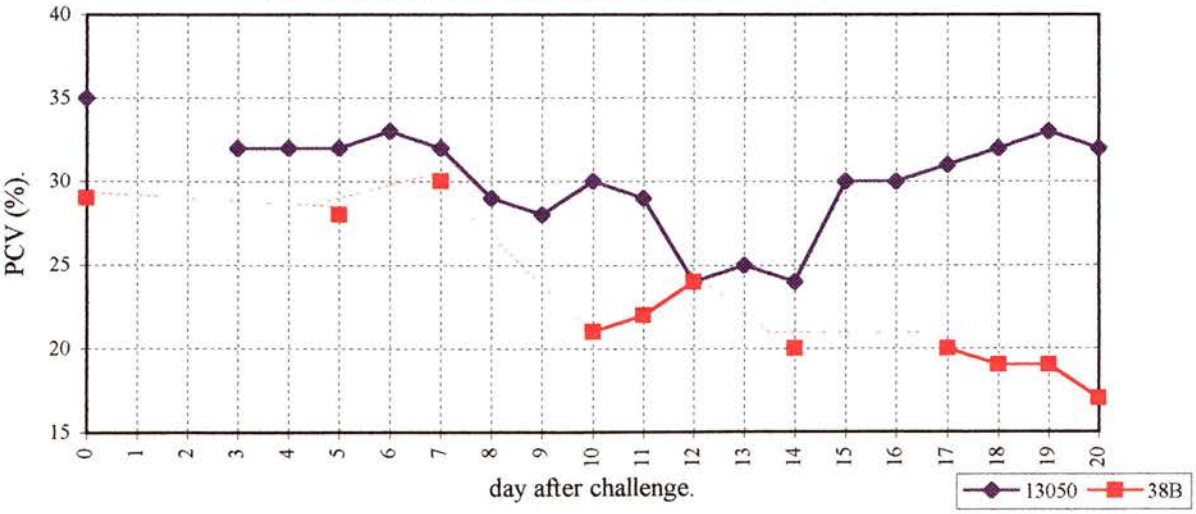
App 5.1.5

PCVs of group L after sporozoite challenge.



App 5.1.6.

PCVs of control animals after sporozoite challenge.

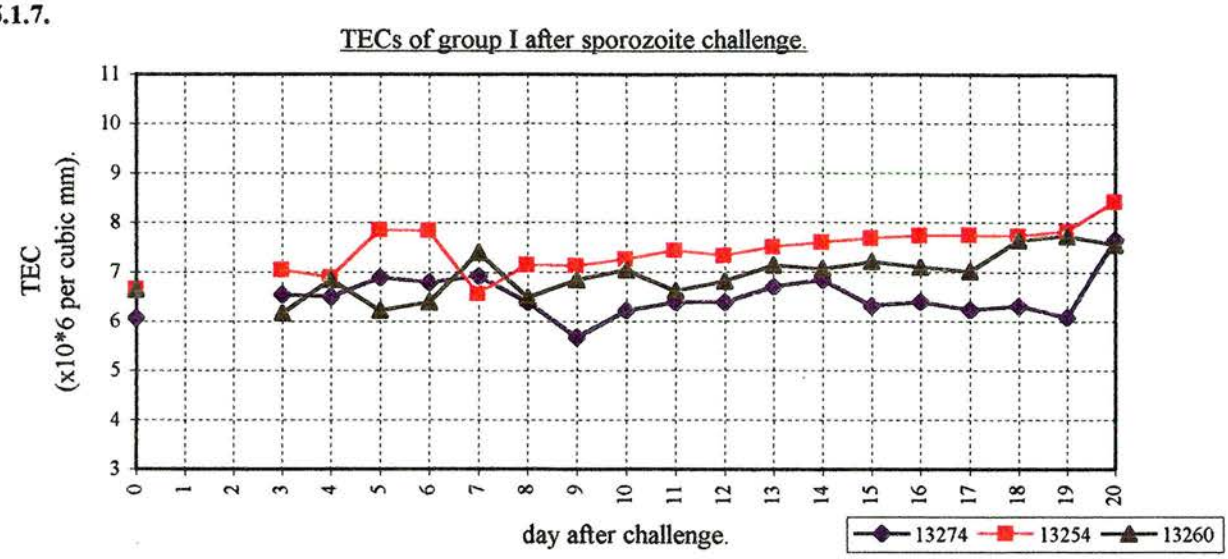


App 5.1.7. **TEC data from animals of group I post challenge.**

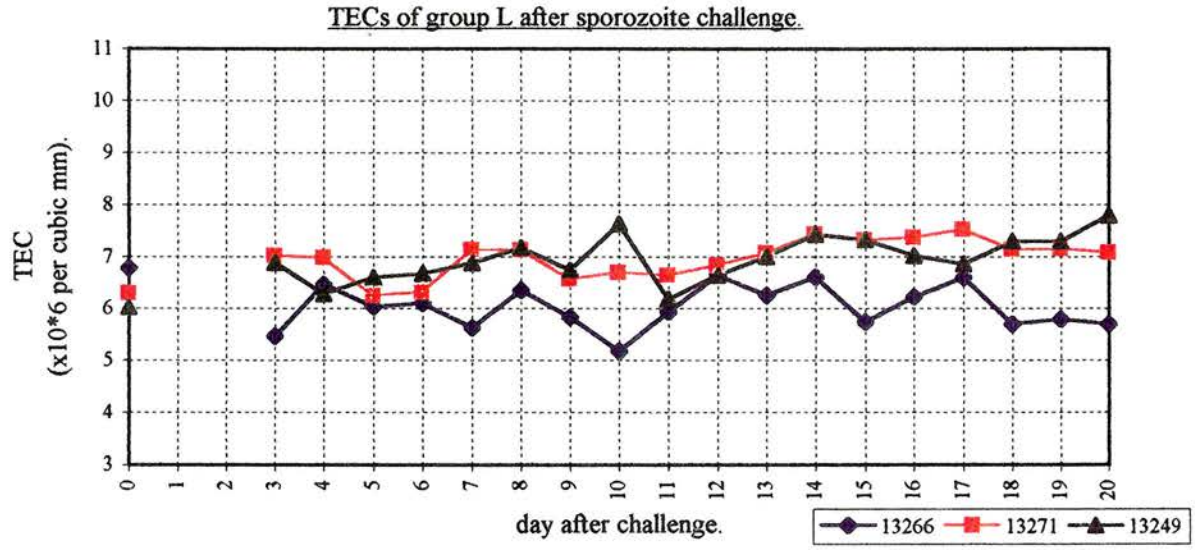
App 5.1.8. **TEC data from animals of group L post challenge.**

App 5.1.9. **TEC data from control animals post challenge.**

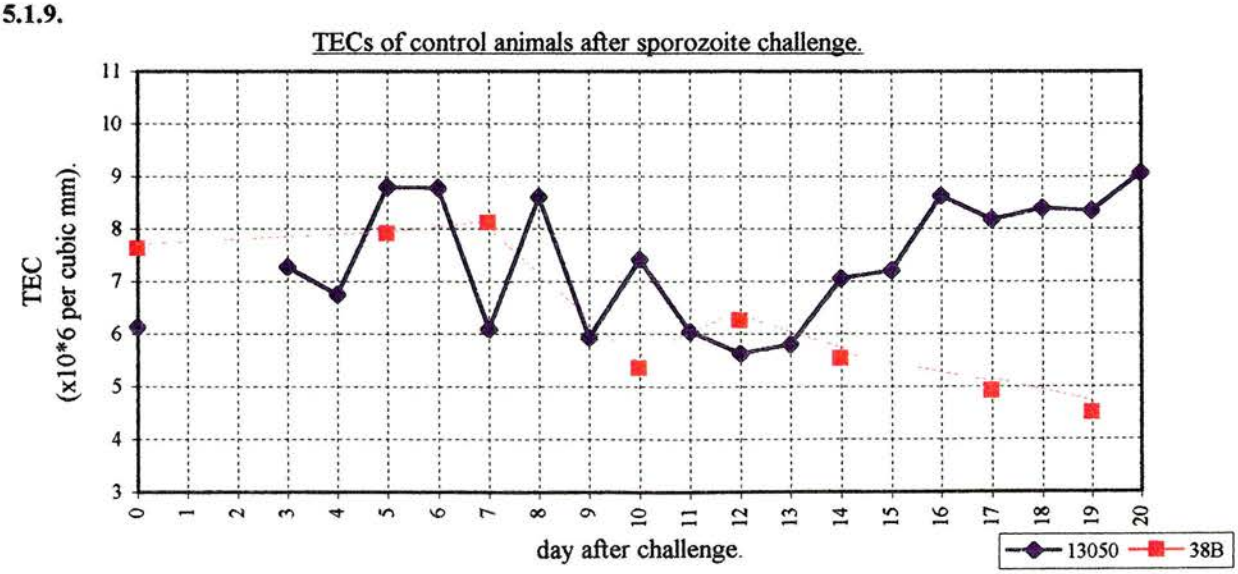
App 5.1.7.



App 5.1.8.



App 5.1.9.



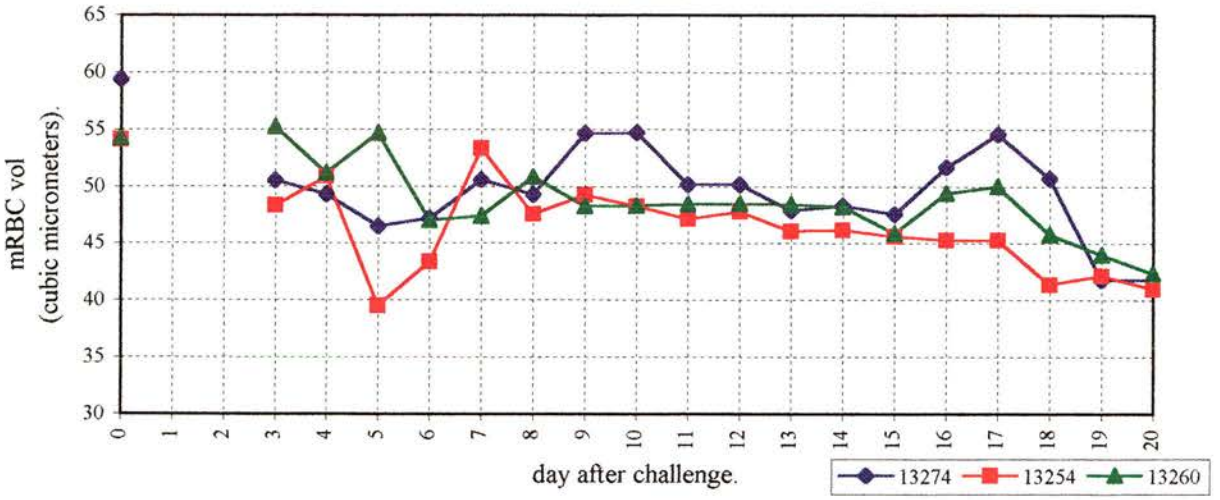
App 5.2.0. **Mean RBC volume data from animals of group I post challenge.**

App 5.2.1. **Mean RBC volume data from animals of group L post challenge.**

App 5.2.2. **Mean RBC volume data from control animals post challenge.**

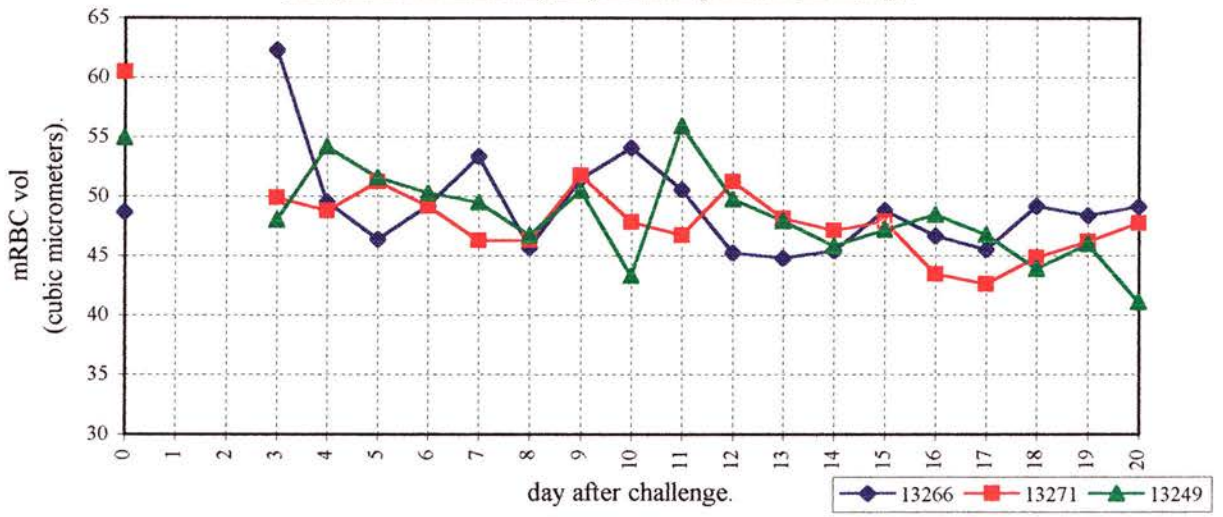
App 5.2.0.

Mean RBC volume of group I after sporozoite challenge.



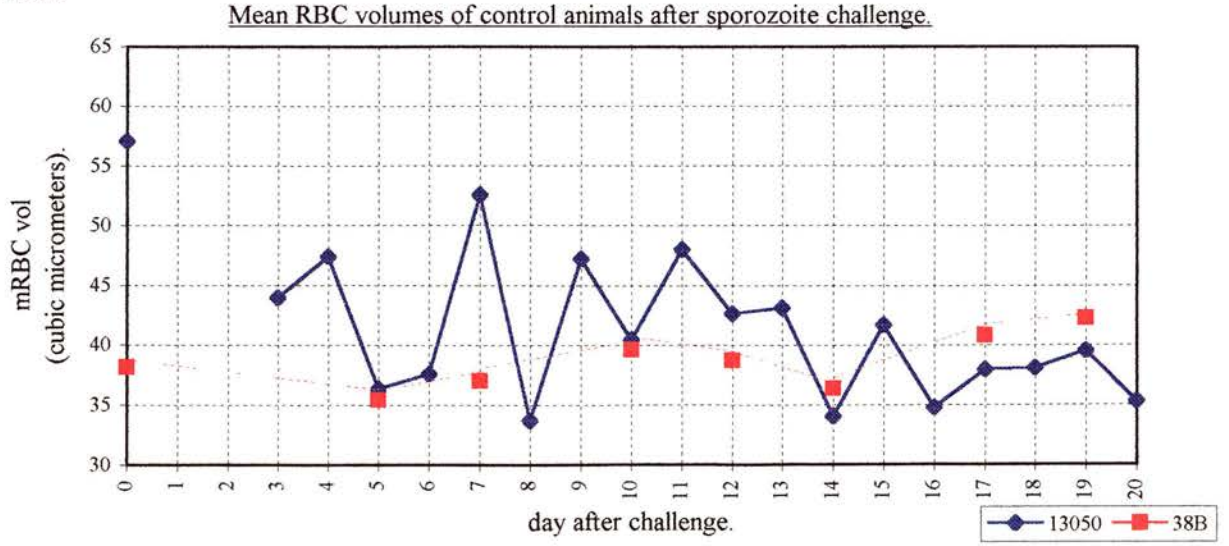
App 5.2.1.

Mean RBC volumes of group L after sporozoite challenge.



App 5.2.2.

Mean RBC volumes of control animals after sporozoite challenge.



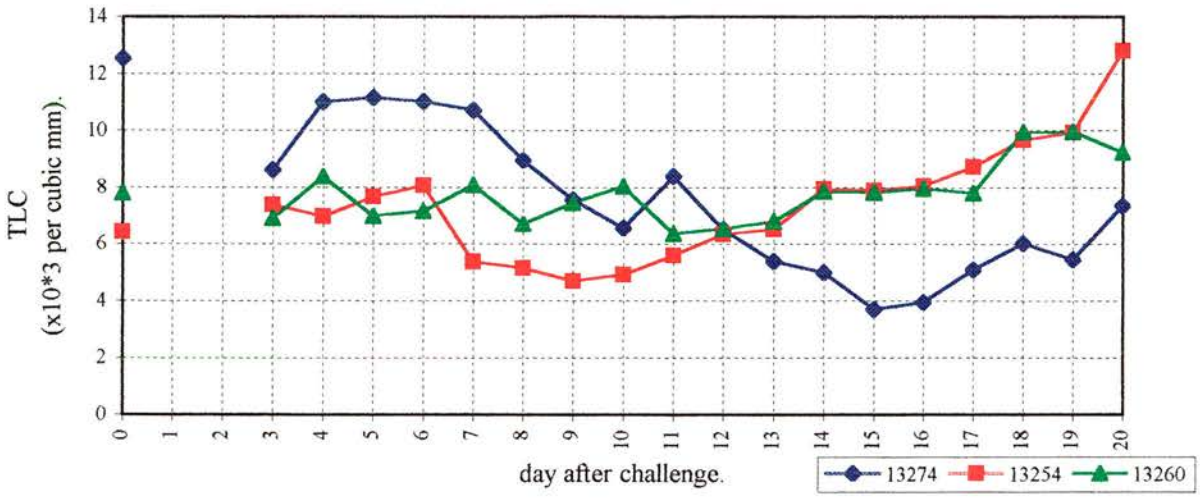
App 5.2.3. **TLC data from animals of group I post challenge.**

App 5.2.4. **TLC data from animals of group L post challenge.**

App 5.2.5. **TLC data from control animals post challenge.**

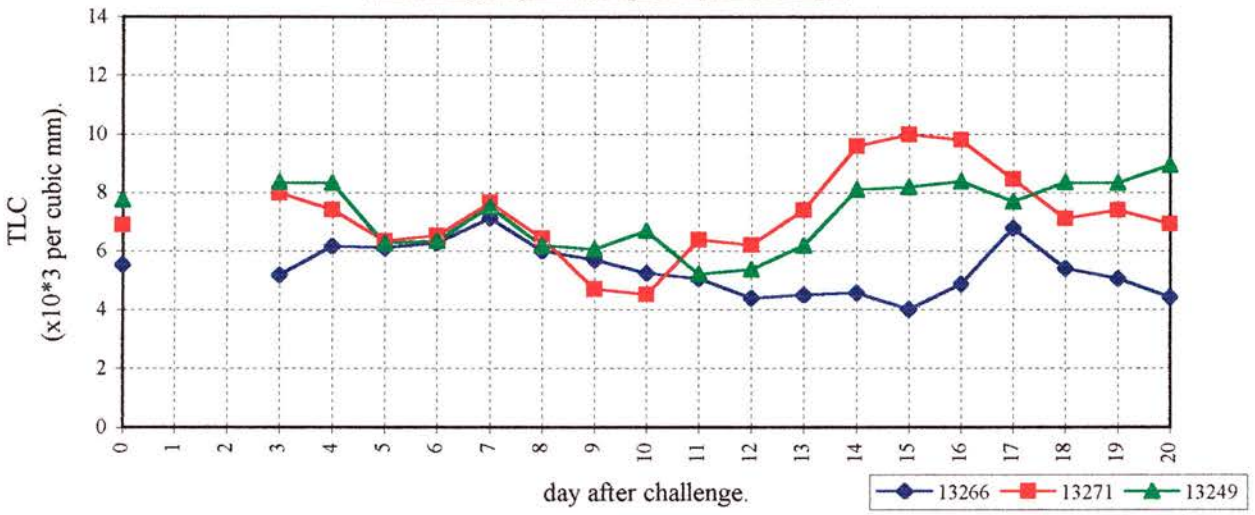
App 5.2.3.

TLCs of group I after sporozoite challenge.



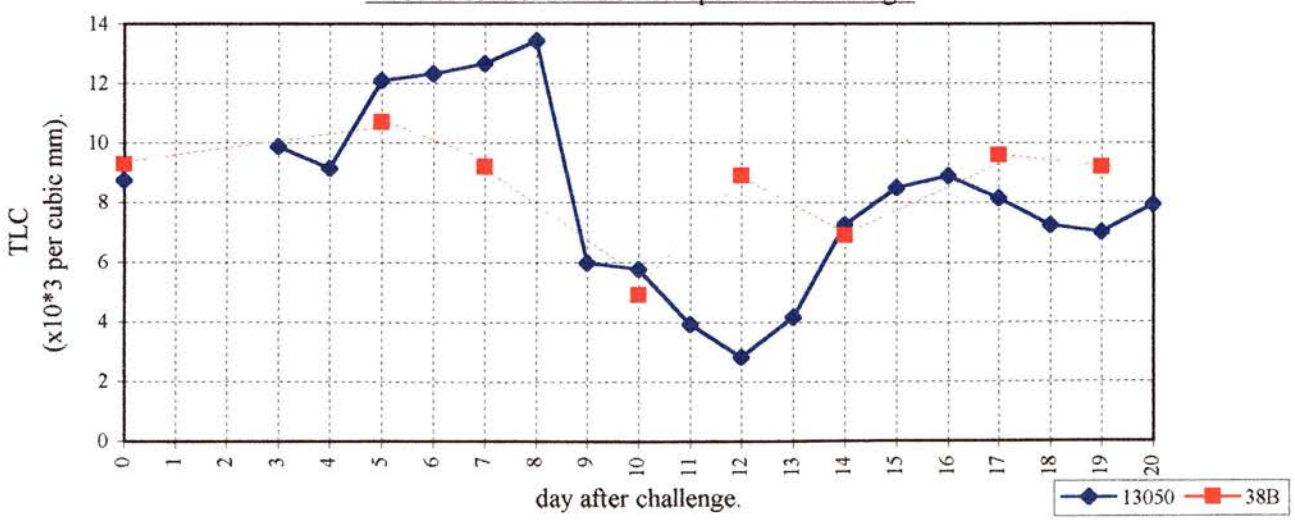
App 5.2.4.

TLCs of group L after sporozoite challenge.



App 5.2.5.

TLCs of control animals after sporozoite challenge.



Abbreviations

Abbreviation	Full form
[^{3}H]dThd	tritiated thymidine
ACD	acid citrate dextrose
APC	antigen presenting cell
App	Appendix
BoLA	bovine lymphocyte antigens
BPA	bovine plasma albumin
bp	base pairs
CD	cluster of differentiation
cDNA	complementary DNA
Con A	concanavalin A
cpm	counts per minute
CTL	cytotoxic T lymphocytes
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytidine triphosphate
dGTP	deoxy guanosine triphosphate
dTTP	deoxy thymidine triphosphate
DMSO	dimethyl sulfoxide
DLN	draining lymph node
EDTA	ethylene di-amino tetra-acetic acid
FACS	fluorescence activated cell scanner
F(ab')$_2$	antibody fragment
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
GAM	goat anti-mouse
GUTS	ground up tick supernate
HPLC	high performance liquid chromatography
IEF	isoelectric focusing
IFAT	indirect fluorescent antibody test

IFN	interferon
Ig	immunoglobulin
IL	interleukin
kD	kilodalton
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	magnetic antibody cell sorter
mCi	millicurie
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
mM	millimole
mRBCv	mean red blood cell volume
mw.	molecular weight
NK	natural killer cell
NRS	normal rabbit serum
PBM	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PCV	packed cell volume
PE	phycoerythrin
PMT	photomultiplier tube
RAM	rabbit anti-mouse
RBC	red blood cells
RFLP	restriction fragment length polymorphism
spp.	species
SSC	side scatter
TE	tick equivalent
TC	tissue culture
TBE	tris-borate-EDTA solution.
TEC	Total erythrocyte count
TLC	Total leucocyte count
Th	T helper

TNF	tumour necrosis factor
WC	workshop culture

Publications.

First author.

Brown DJ, Campbell JDM, Russell GC, Hopkins J and Glass EJ. (1995). T cell activation by *Theileria annulata* infected macrophages correlates with cytokine production. *Clinical and Experimental Immunology*. **102**: 507-514.

Co-author.

Campbell JD, Brown DJ, Glass EJ, Hall FR and Spooner RL. *Theileria annulata* sporozoite targets. (1994). *Parasite Immunology*. **16**: 501-505.

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Sutherland IA, Sheils BR, Jackson L, Brown DJ, Brown CGD and Preston PM. (1996). *Theileria annulata*: altered gene expression and clonal selection during continuous *in vitro* culture. *Experimental Parasitology*. **83**: 125-133.

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T cell activation by *Theileria annulata*-infected macrophages correlates with cytokine production

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SUMMARY

A major feature of the pathology induced by *Theileria annulata* is acute lymphocytic proliferation, and this study investigates the mechanisms underlying the intrinsic ability of *T. annulata*-infected monocytes to induce naive autologous T cells to proliferate. Different *T. annulata*-infected clones expressed different but constant levels of MHC class II, varying from $< 1{\cdot}0 \times 10^5$ to $1{\cdot}5 \times 10^6$ molecules/cell, as measured by saturation binding. However, no correlation was found between the level of MHC class II expression and levels of induced T cell proliferation. *Theileria annulata*-infected cell lines and clones were assayed for cytokine mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR). The infected cells assayed produced mRNA specific for IL-1α, IL-1β, IL-6, IL-10 and tumour necrosis factor-alpha (TNF-α), but not IL-2 or IL-4. One clone (clone G) did not produce mRNA for TNF-α. The degree of T cell proliferation induced by infected cells was directly correlated with the amount of mRNA produced for the T cell stimulatory cytokines IL-1α and IL-6, as assessed by a semiquantitative technique. In contrast, cells infected with the related parasite *T. parva* produced mRNA for IL-1α, IL-2, IL-4, IL-10 and interferon-gamma (IFN-γ). Since *T. parva*-infected cells also induce naive autologous T cell proliferation, it seems likely that the production of IL-1α by cells infected with either parasite is a major signal for the induction of non-specific T cell proliferation.

Keywords *Theileria annulata* bovine cytokine macrophage MHC class II

INTRODUCTION

Theileria annulata is a tick-transmitted protozoan parasite of cattle and causes the severe lymphoproliferative disease, tropical theileriosis, endemic in Southern Europe, North Africa, Southern Russia, India and the Middle East. *In vitro*, infective sporozoites invade MHC class II^+ cells, particularly cells of the monocyte/macrophage lineage which are able to form continuously growing cell lines *in vitro* [1,2]. Theileria species appear to be unique among intracellular protozoan parasites, as no other species is known to exist within the cytoplasm of macrophages and induce their replication. Upon infection, cells acquire a uniform phenotype irrespective of their origin [3]. Infected cells can act as professional antigen-presenting cells (APC), capable of presenting exogenous antigens to $CD4^+$ T cells [4]. However, these cells are also capable of activating naive autologous T cells in the absence of exogenous soluble antigen in a contact-dependent manner [5]. This property of infected cells has recently been shown to play a major role in disease pathogenesis, bypassing normal T cell–APC interactions within draining lymph nodes [5].

MHC class II-restricted antigen presentation and cytokine secretion are essential for APC function (at least in the activation of $CD4^+$ cells), and alteration of either component may play an important role in the altered APC function of infected cells and thus pathogenesis.

In this study we have generated clonal *T. annulata*-infected cell lines, quantified the levels of expression of MHC class II molecules upon them, and examined whether there is a correlation between MHC class II expression, cytokine production of infected cells, and T cell stimulatory ability.

The related parasite *T. parva* infects T cells and causes a disease known as East Coast Fever which is endemic to the East coast of Africa [6]. There are a number of similarities between these two parasites. *Theileria parva*-infected cells are also MHC class II^+ and also induce T cell proliferation [7]. Therefore the cytokines produced by a *T. parva*-infected cell line were also investigated and compared with the cytokines produced by *T. annulata*-infected cells.

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MATERIALS AND METHODS

Theileria annulata-*infected cell lines and clones*

A *T. annulata* (Ankara strain)-infected cell line (*Theileria annulata* 12929 (*T.a* 12929)) [8] was established from $CD14^+$ peripheral blood monocytes (PBM) and macrophages of a 6-month-old Friesian calf as previously described [3,4,7]. Clonal cell lines were produced from this line by soft agar cloning of the *T.a* 12929 cell line generated above [9]. At a cell density of 2×10^5 cells/well, cells were then transferred to 10-ml Nunc tissue culture flasks and treated in an identical manner to standard *T. annulata* cultures [10].

Theileria parva *cell line*

The *T. parva* cell line (B641L4.14 TpM) was used in this work (a kind gift from ILRI).

Quantification of MHC class II expression

Cell cultures were passaged the day before use to maintain the cells in exponential growth phase. The MoAb used during this assay was the rat anti-ruminant MHC class II (DRβ-specific) MoAb SW73.2 [11]. $F(ab')_2$ fragments of MoAb SW73.2 were iodinated with ^{125}iodine (Amersham Int., Aylesbury, UK) using the Iodobeads iodination protocol (Pierce, Rockford, IL) [12]. The level of expression of MHC class II molecules was measured by saturation binding using ^{125}I-labelled SW73.2 $F(ab')_2$ as previously described [13,14], except that cells were labelled on ice for 30 min before unbound $F(ab')_2$ was separated from the cells by washing with aliquots of PBS. The results obtained from assays of the cloned bovine B cell lymphoma line BL20 were identical to those previously described [15].

Flow cytometric analysis

Flow cytometric analysis of *T. annulata*-infected cells was performed to assess the expression of MHC class I and class II molecules [3]. This analysis was performed using the MoAbs SW73.2 and IL-A21 [16] which recognize monomorphic determinants on bovine MHC class II molecules and the anti-bovine MHC class I MoAb IL-A19 [17].

Proliferation assays

T cell proliferation induced by the infected cell lines was measured as previously described [4]. The *T. annulata*-infected cells used in these experiments were passaged 24 h before use; 2 ml of the cell culture were added to 8 ml of fresh medium. Briefly, autologous naive peripheral blood mononuclear cells (PBMC; 4×10^5/well) were incubated with irradiated (cells exposed to 75 Gy) autologous *T. annulata*-infected cells (4×10^4/well). Assays were harvested on day 5 (the day of maximum proliferation [5]) following a 6-h pulse of tritiated thymidine (^{3}H-TdR) (Amersham). Proliferation was measured by liquid scintillation counting using a Wallac 1450 Microbeta. Background ct/min were < 1500 for irradiated PBMC alone and < 2500 ct/min for irradiated *T. annulata*-infected cell lines alone.

Reverse transcription-polymerase chain reaction analysis of cytokine transcripts

Total cell mRNA was isolated from various *T. annulata* cell lines and clones using RNAzol B (Biogenesis, Poole, UK) according to the manufacturer's instructions. Briefly, 10^7 cells were suspended in 1 ml of RNAzol B and the RNA extracted using ice-cold chloroform/isoamylalcohol (24:1). The RNA was precipitated at -20°C in isopropanol, followed by two washes in 75% ethanol. RNA was then dried under vacuum, resuspended in a suitable volume of sterile dH_2O, and the yield determined by spectrophotometry (OD taken at 260 nm). Samples of 5 μg RNA were used for reverse transcription of cDNA using the SuperScript preamplification system (GIBCO BRL, Paisley, UK) with oligo dT as a primer, according to the manufacturer's instructions. Polymerase chain reaction (PCR) reactions were performed using 2 μl of the resulting cDNA. Primers specific for bovine cytokine sequences (Table 1) were used to amplify cytokine cDNA, during a 30 cycle PCR programme (Taq polymerase and buffers from GIBCO). Primers specific for β-actin, IL-2 and IL-4 were used as previously described [18]. The products of the reverse transcription (RT)-PCR reactions were visualized by UV transillumination, following electrophoresis on a 2% agarose (Sigma, type 1-A; Poole, UK)/1X TBE gel containing 0·1 μg/ml ethidium bromide.

Table 1. Cytokine primer sequences used in this study

mRNA	Primer direction	Size, bp	5′-sequence-3′
β-actin	+	288	CTGGCACGACACCTTCAACGAG
	−		AGCCAAGTCCAGACGCAGGATG
IL-1α	+	332	TCACCGATGATGACCTGGAAGCC
	−		GATTTTGGGTGTCTCAGGCATCTCC
IL-1β	+	432	CCGACGAGTTTCTGTGTGACGCACC
	−		CGAAAATGTCCCAGGAAGACGGGC
IL-6	+	655	ATGAACTCCCGCTTCACAAGC
	−		TACTTCATCCGAATAGCTCTC
IL-10	+	733	GCTCAGCACTACTCTGTT
	−		GTTCACAGAGAAGCTCAGT
TNF-α	+	500	CTCAGGTCATCTTCTCAAGCC
	−		CAGGGCGATGATCCCAAAGTAGACC
IFN-γ	+	531	GGAGCTACCGATTTCAACTACTCCG
	−		GCAGGCAGGAGGACCATTACG

Primer orientation (+ or −) is given in relation to the direction of transcription. The β-actin, IL-2, and IL-4 sequences have already been reported [18].

Quantification of mRNA

Limiting step PCR was performed to ascertain the relative expression levels of the various cytokine mRNAs. The PCR protocol remained the same apart from the number of cycles used to amplify the cytokine cDNA. Cycle numbers ranged from 20 to 30, in increments of two cycles. Comparison of the cycle at which the PCR products became visible was used to assess the relative expression of the cytokine mRNA species. β-actin expression was used as an internal standard for these reactions.

Restriction digest analysis of PCR products

The identity of the PCR products was determined by restriction enzyme analysis, using five enzymes: Taq 1 (Pharmacia, Uppsala, Sweden), Pvu II (Boehringer-Mannheim, Lewes, UK), Bgl II (Boehringer-Mannhiem), Hae III (New England Biolabs, Herts, UK), Pst 1 (Pharmacia). PCR product (15 μl)

was digested with the selected restriction enzymes. The fragments obtained after digestion were visualized as in Fig. 3, and their sizes matched those expected from the known sequences.

Statistical analysis of MHC class II quantification and T cell proliferation assays

The data obtained from MHC class II quantification and T cell proliferation assays were assessed using Student's *t*-test. Statistical analysis was carried out on the MINITAB statistical program.

RESULTS

Preliminary flow cytometric analysis showed that the uncloned parent line (*T.a* 12929) had a wide distribution of MHC class II expression (Fig. 1). Fourteen clones were produced by soft agar cloning. Initial analysis of these clones showed them to express varying levels of MHC class II. Three clones (clones G, I and L) were selected which expressed levels of MHC class II molecules ranging from below, to approaching that of the original culture (*T.a* 12929). Uninfected monocytes, the parental population *T.a* 12929 and clonal lines G, I and L derived from this cell line were assayed for the amount of MHC class II expressed at the cell surface (Fig. 1 and Table 2).

Flow cytometric analysis of MHC class I and II expression

The parent line and the clones all showed a normal distribution of MHC class I expression with a small range (Fig. 1). In contrast, the expression of MHC class II by the parent line and the clones was not normally distributed, even though these cells were part of a clonal culture and the range of expression of MHC class II was much greater than that of MHC class I. All of the clones expressed less MHC class II than the parent line, and clone G expressed higher MHC class II levels than the other clones.

Table 2. MHC class II cell surface expression on *T.a* 12929 and cloned cell lines

Cell line	No. class II molecules/cell	Range
Uninfected monocytes	$2{\cdot}32 \times 10^5$	ND
clone I	$8{\cdot}0 \times 10^4$	$8{\cdot}0 \times 10^4$–$1{\cdot}0 \times 10^5$
clone L	$1{\cdot}0 \times 10^5$	$1{\cdot}0 \times 10^5$–$2{\cdot}0 \times 10^5$
clone G	$1{\cdot}0 \times 10^6$	$1{\cdot}0 \times 10^6$–$2{\cdot}0 \times 10^6$
T.a 12929	$2{\cdot}4 \times 10^6$	$2{\cdot}4 \times 10^6$–$2{\cdot}5 \times 10^6$

The number of MHC class II molecules expressed per cell given are the result of a representative experiment. The range given is for three experiments, except for the uninfected monocyte sample (ND).

Saturation binding studies

Table 2 shows the data produced from saturation binding studies. The parent cell line expressed higher levels of MHC class II than any of the clonal populations. Clone G showed the highest expression of MHC class II of the clones, whilst clones I

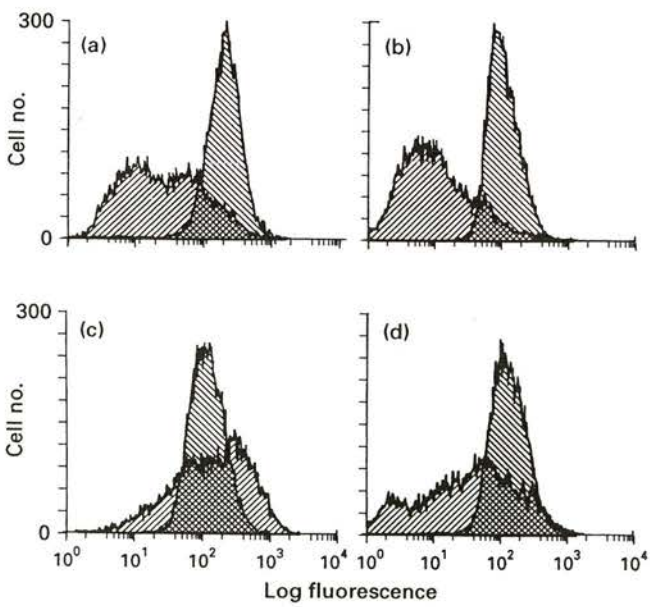

Fig. 1. Flow cytometric analysis of MHC class I and II expression from (a) clone I, (b) clone L, (c) clone G, and (d) *T.a* 12929. Profiles include MHC class I profile (▨) cells stained with the anti-bovine MHC class I MoAb IL-A19, and a profile of the MHC class II expression (▨), cells stained with the anti-bovine MHC class II MoAb IL-A21.

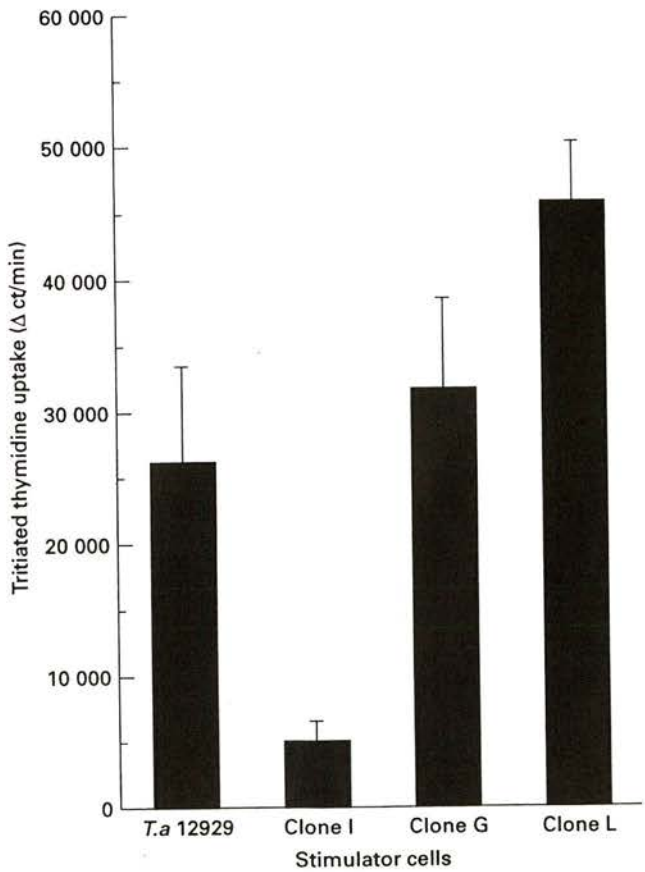

Fig. 2. Proliferation assays using the cell lines (*T.a* 12929, clones I, G and L) to stimulate freshly isolated peripheral blood monocytes (PBM). Data shown as Δct/min (Δct/min = ct/min of proliferating stimulated T cells – (ct/min of *T. annulata*-infected cells alone + ct/min of PBM alone)).

and L expressed similar levels of MHC class II. Statistical analysis showed that the variation in MHC class II expression within triplicate samples of the parent line and clones was not statistically significant. However, the expression of MHC class II by the parent line and clone G was shown to be significantly different from clones I and L ($P \leqslant 0{\cdot}001$).

Proliferation of naive T cells

Clonal cell lines and the uncloned parent line (*T.a* 12929) were used in proliferation assays to assess the level of non-specific T cell proliferation induced when naive autologous T cells were incubated with each clone and proliferation was measured on day 5 (Fig. 2). Clone L induced the highest net levels of proliferation (45 565 ± 4504 ct/min), which was significantly different from the proliferation induced by the parent line and clone I ($P \leqslant 0{\cdot}005$). Both clone G and the parent line induced similar levels of proliferation, lower than clone L (31 588 ± 6792 and 26 091 ± 7326 ct/min, respectively). In contrast, clone I induced extremely low proliferation (4908 ± 1527 ct/min), significantly lower than all other infected cells ($P \leqslant 0{\cdot}005$). No correlation was found between the expression of high levels of MHC class II on the surface of infected cells and the levels of proliferation induced by the parent line and the clones.

Cytokine production from infected cells

The possibility that the production of T cell stimulatory cytokines by the infected cells led to the activation of naive T cells was investigated using RT-PCR analysis of cytokine mRNA. Restriction enzyme analysis of the PCR products showed that the sequence of the RT-PCR products matched the known bovine cytokine sequences (Fig. 3). The cytokines produced by the uncloned parent line (*T.a* 12929) and a *T. parva*-infected cell line are shown in Fig. 4a and b, respectively. The *T. annulata*-infected cell line and clones expressed mRNA specific for the monocyte and macrophage-associated cytokines: IL-1α, IL-1β, IL-6, IL-10 and tumour necrosis factor-alpha (TNF-α). Clone G did not produce detectable levels of TNF-α. The parent line was also found to produce interferon-gamma (IFN-γ) mRNA on two out of five occasions. None of the clones was found to produce mRNA specific for IFN-γ. The *T. parva*-infected cell line expressed the T cell-associated cytokines IL-2, IL-4 and IFN-γ, as well as IL-1α and IL-10.

Limiting cycle PCR was used to investigate the relative amounts of each cytokine mRNA produced by each cell line (Fig. 5 and Table 3). Figure 5 shows the levels of expression of mRNA specific for β-actin, IL-1α, IL-1β and IL-6 by the parent line and clones at different PCR cycle numbers (between 20 and 24 cycles). Table 3 shows the cycle numbers at which the cytokine PCR products were detectable. PCR amplifies DNA templates exponentially, therefore a product which becomes visible at 20 cycles results from a much more abundant source of mRNA than a product visible at higher numbers of cycles. Clone L produced the highest levels of mRNA for IL-1α, IL-1β, IL-6 and TNF-α (visible at 20 cycles) and IL-10 mRNA visible at 24 cycles. The parent line and clones G and I expressed much lower levels of these mRNAs, with no detectable mRNA for IL-10 or TNF-α being expressed by clone G.

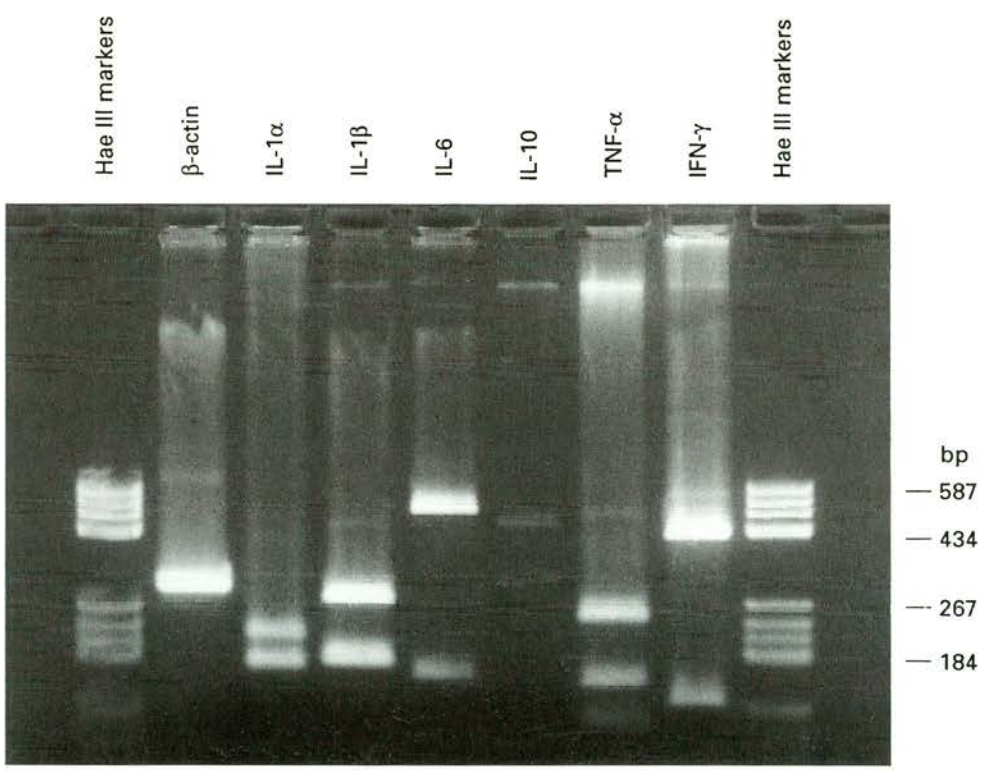

Fig. 3. Restriction enzyme analysis of the cytokine reverse transcription-polymerase chain reaction (RT-PCR) products. The enzymes used and fragments expected are as follows, IL-1α (Taq 1) 161 bp/204 bp, IL-1β (Pvu II) 169 bp/263 bp, IL-6 (Bgl II) 149 bp/477 bp, IL-10 (Hae III) 306 bp/439 bp, tumour necrosis factor-alpha (TNF-α) (Pvu II) 99 bp/163 bp/234 bp, IFN-γ (Pst 1) 114 bp/425 bp.

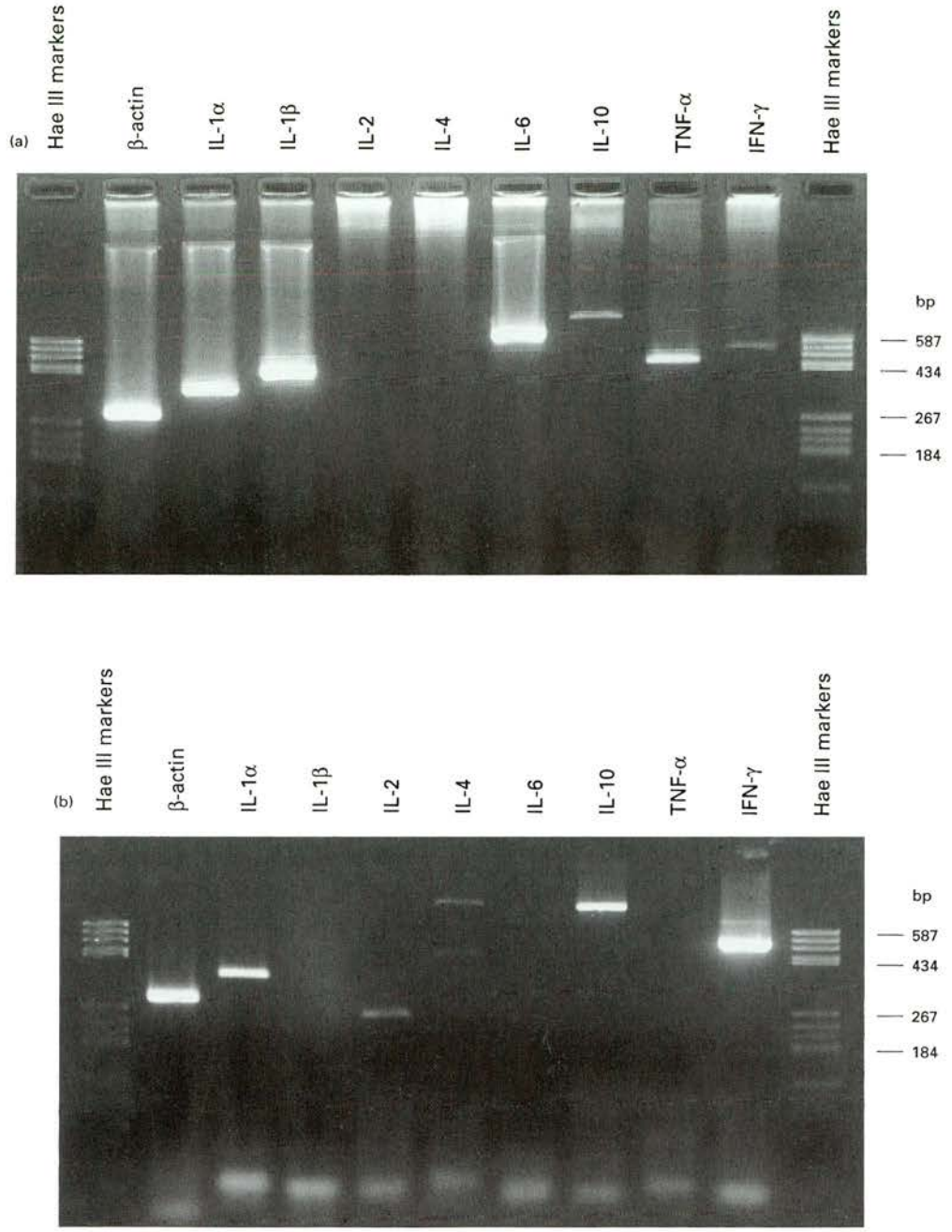

Fig. 4. (a) Cytokine gene expression determined by reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA from the parental line (*T.a* 12929). Other *T. annulata*-infected cell lines tested so far also expressed mRNA for the listed cytokines except IFN-γ (data not shown). The analysis of mRNA from the *T.a* 12929 cell line was carried out on five separate occasions, but IFN-γ mRNA was detected on only two occasions. (b) Cytokine gene expression in a *T. parva*-infected line.

DISCUSSION

Our previous studies have shown that *T. annulata*-infected cells have enhanced APC function [4] and also induce aberrant T cell activation and proliferation *in vitro* and *in vivo* [4,5]. Originally we attributed this to the high levels of MHC class II expressed on the surface of infected cells [4]. Our results here indicate that this is unlikely to be the case. Interestingly, although all of the uncloned *T. annulata*-infected cell lines which we have examined to date expressed higher average levels of MHC class II molecules than the original uninfected $CD14^+$ monocytes, we were nevertheless able to clone several cell lines which expressed fewer MHC class II molecules on a per cell basis than the original uninfected cells. Also it can be seen from the flow cytometry profiles in Fig. 1 that the expression of MHC class II molecules by the clones is not normally distributed. This dysregulation does not appear to alter MHC class I gene expression, suggesting that the parasite within infected cells induces a highly specific dysregulation of MHC class II expression.

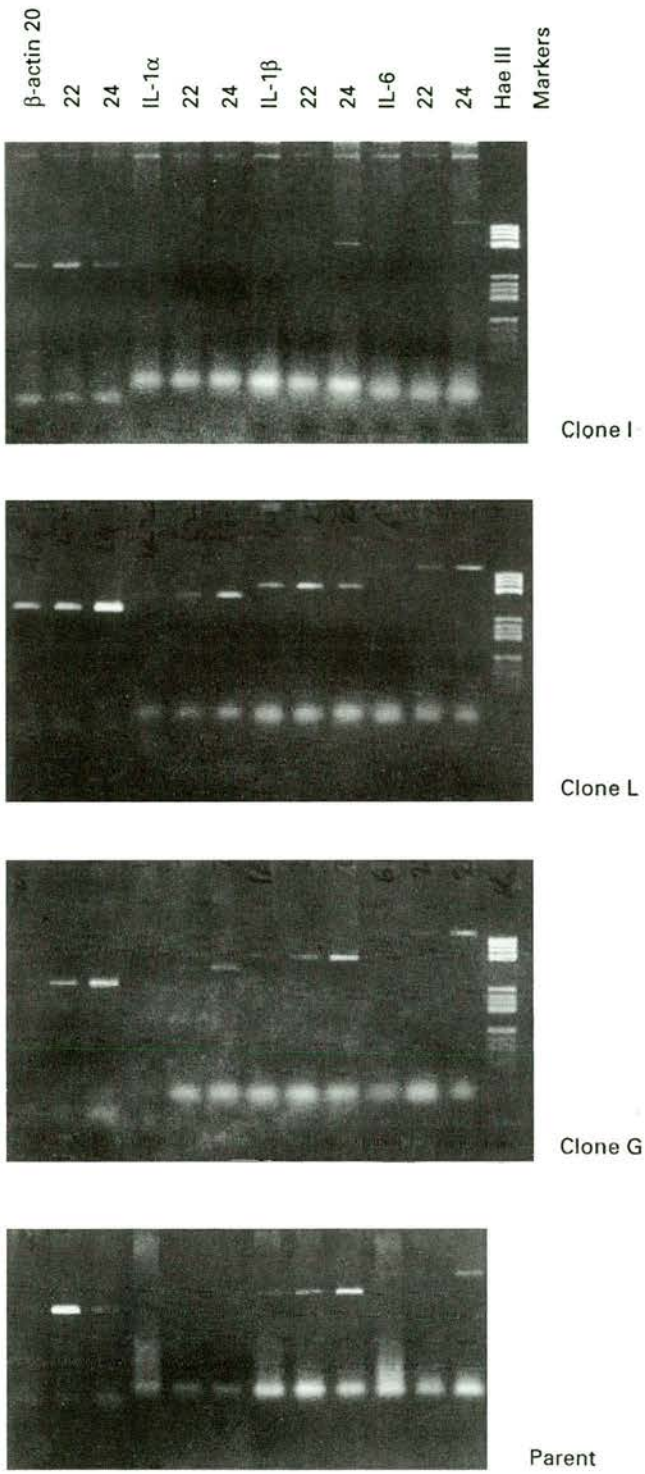

Fig. 5. Limiting cycle reverse transcription-polymerase chain reaction (RT-PCR) analysis of β-actin, IL-1α, IL-1β and IL-6 expression for clones I, G, L and *T.a* 12929.

Table 3. Cytokine gene expression assayed by limiting cycle polymerase chain reaction (PCR)

Cytokine	*T.a* 12929	Clone I	Clone L	Clone G
β-actin	20	20	20	20
IL-1α	24	26	20	24
IL-1β	20	24	20	20
IL-6	24	24	20	22
IL-10	30	28	24	NP
TNF-α	22	28	20	NP
IFN-γ	30	NP	NP	NP

The cycle number at which the PCR product becomes visible is given above. NP, The product was not visible after 30 PCR amplification cycles.

In this study we have shown that cells infected with *T. annulata* constitutively produce mRNAs for a number of macrophage-associated cytokines, including IL-1α, IL-1β, IL-6, IL-10 and TNF-α. By isolating a number of *T. annulata*-infected clones and carrying out limiting cycle RT-PCR, we were able to show that the levels of T cell proliferation induced by infected cells correlated with the levels of expression of the T cell stimulatory cytokines, IL-1α, IL-1β and IL-6. Clone L, which expressed the highest levels of these cytokines, induced the highest levels of T cell proliferation. In contrast, clone G, which expressed lower levels of these cytokines, induced less proliferation, despite expressing 15 times more MHC class II molecules than clone L, and clone I, which expressed the lowest levels of these cytokine mRNA species, induced the lowest levels of naive autologous T cell proliferation.

A T cell line infected with the related parasite *T. parva* produced a different range of cytokine mRNAs, including IL-1α, IL-2, IL-4, IL-10 and IFN-γ. The production of IL-1α by the *T. parva*-infected cell line is surprising, as T cells do not normally produce this cytokine [19]. Cells infected with either of these parasites induce contact-dependent proliferation in naive autologous T cells [5,7], suggesting that these parasites share a common mechanism for the stimulation of T cells. The presence of IL-1α mRNA in both types of infected cells suggests that this cytokine is the most important in the induction of T cell proliferation by infected cells.

The induction of T cell proliferation also depended on the state of growth of the infected cells. If the infected cell lines or clones were not passaged 24 h before the non-specific proliferation assay was initiated, the levels of T cell proliferation observed were considerably reduced (data not shown). This suggests that the ability of the infected cells to induce T cell proliferation may relate to the growth phase of the cell, as passaging the culture 24 h earlier stimulated growth of infected cells.

We have previously shown that non-specific activation of T cells also occurs *in vivo* [5]. During *T. annulata* infection, T cell blasts are found surrounding foci of macroschizont infected cells in the lymph nodes draining infection. These cells are unlikely to be stimulated by classical antigen-presentation mechanisms, as they appear within 2 days post-infection. Our data suggest that the primary mechanism by which *T. annulata* induces T cell activation *in vivo* is through the production of T cell stimulatory cytokines by the infected cells.

Cells infected with *T. annulata* also produce high levels of inflammatory cytokines, especially TNF-α. This cytokine is a potent inducer of fever and has also been linked to the

production of anaemia, muscle wasting and necrosis [20,21]. These symptoms are observed in acute cases of tropical theileriosis. Although the anaemia and fever observed have been linked to the formation of piroplasms in erythrocytes, followed by the lysis of the infected cells, there are documented cases where infections have proved fatal before the detection of piroplasms [22]. In addition, the cell line infected with *T. parva* does not produce mRNA specific for TNF-α, and *T. parva* infections are not characterized by high levels of anaemia [23]. We suggest that the production of TNF-α by the *T. annulata*-infected cells directly causes the pathological reactions observed during *T. annulata* infections.

Analysis of the parent cell line showed that IFN-γ mRNA was produced on two out of five occasions, but none of the clones or other cell lines assayed was found to produce mRNA specific for this cytokine. Although high levels of IFN-γ are detected in the efferent lymph during *T. annulata* infection [24], the IFN-γ produced presumably originates mainly from *T. annulata*-stimulated T cells and not from the infected cells themselves. We have shown that T cells stimulated by *T. annulata*-infected cells *in vitro* produce IFN-γ mRNA (J. D. M. Campbell, manuscript in preparation). The production of IFN-γ by activated T cells may exacerbate the infection by stimulating macrophages to produce more TNF-α and so increase fever.

In conclusion, infection of leucocytes with the related parasites, *T. annulata* and *T. parva*, induces the secretion of numerous cytokines by the infected cells. *Theileria annulata* induces the production of various T cell-stimulatory and inflammatory cytokines which may be responsible for the immunopathological symptoms observed during infection. Also, the production of a range of antagonistic cytokines such as IL-1 and IL-10 *in vivo* [25,26] may reduce the efficiency of immune responses against infected cells, whereas the presence of certain cytokines (such as IL-1 and IFN-γ) may promote the development of infected cells. Alteration of cytokine expression is also seen in leishmania-infected macrophages [27,28]. However, this parasite appears to down-regulate production of several cytokines, including IL-1α, IL-6, IL-10 and IL-12 [27,28].

We plan to investigate the cytokine profiles of attenuated and virulent lines as the levels of cytokine expression may relate to the pathology induced by these cell lines. The low levels of cytokine mRNA and low T cell stimulatory ability found in clone I and the lack of TNF-α produced by clone G may mean that these are possible candidates for use as cell line vaccines.

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Brief communication

Theileria annulata sporozoite targets

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SUMMARY

Bovine peripheral blood mononuclear cells (PBM) infected in vitro *with* Theileria annulata *sporozoites have previously been characterized as MHC class II$^+$ mature macrophages. The ability of* T. annulata *sporozoites to infect different subpopulations of MHC class II$^+$ bovine monocytes was investigated. Cells were labelled with monocyte specific monoclonal antibodies (MoAb) and isolated using magnetic cell sorting (MACS). Sporozoites infected both immature and mature monocytes, but more readily infected the mature population. A potential ligand for sporozoite entry is the elastin receptor which is expressed mainly on the immature population of monocytes and not on B cells or T cells.* T. annulata *sporozoites infected elastin receptor positive and negative cell populations equally well. Infected immature cells lost the expression of elastin receptors and the immature marker, subsequently expressing the mature marker. All monocytes lost the expression of CD14 (the LPS receptor) upon infection with sporozoites. The infection of specific populations and subsequent alterations in phenotype may alter the function of these cells and play an important role in disease pathogenesis.*

Keywords T. annulata *monocytes, FACS analysis, MACS separation, limiting dilution*

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INTRODUCTION

Theileria annulata is a tick transmitted protozoan parasite which affects cattle in Southern Europe, North Africa, India, the Middle East and Southern Russia, causing tropical theileriosis, a life threatening disease. We have shown *in vitro* that sporozoites invade major histocompatibility complex (MHC) class II$^+$ cells, particularly cells of the macrophage/monocyte lineage and also, though much less efficiently, B cells (Spooner *et al.* 1989, Glass *et al.* 1989). Whatever the origin of the initial infected cell, all cell lines apparently had a similar phenotype as judged by the available markers. Infected cells were nonspecific esterase$^+$ plus MHC class II$^+$ and surface IgM$^-$ and T cell marker$^-$ (Spooner *et al.* 1988, 1989, Glass & Spooner 1990). The monoclonal antibody (MoAb) IL-A24 detects a determinant on macrophages associated with antigen presentation (Ellis *et al.* 1987). This ligand is also expressed upon sporozoite infected cells, which can present antigen via MHC class II (Glass & Spooner 1990).

Distinct ligands upon the surface of the target cells may be required for sporozoite attachment and invasion. Identification of these ligands and further characterization of the primary cells which sporozoites infect could be of prime importance in understanding the pathogenesis of this disease and the variation seen in responses to currently used cell line vaccines (Ouhelli *et al.* 1989).

This study extends our previous findings and more fully characterises the phenotype of cells infected by *T. annulata*, using three recently available MoAbs to potentially relevant surface antigens. The MoAbs are IL-A109, VPM65 and BCZ.

IL-A109 recognizes monocytes and macrophages and is thought to recognize an antigen analogous to CD64 (FcR1) (MacHugh, McKeever & Goddeeris 1990).

VPM65 recognizes ovine CD14 (VK Gupta, personal communication) and crossreacts with bovine macro-

phages and B cells, although cell sorting using this MoAb isolates only monocytes/macrophages (see below) (D.J.Brown, unpublished observations). In humans, CD14 is a 55kDa glycoprotein expressed as a cell surface molecule on macrophages and a small population of B cells, and acts as a receptor for LPS (reviewed in Zeigler-Heitbrock & Ulevitch 1993).

BCZ recognizes the elastin receptor (Mecham *et al.* 1989). Elastin binds to its receptor via a repeating hexapeptide: Val-Gly-Val-Ala-Pro-Gly (VGVAPG) (Mecham *et al.* 1989). Recently, the gene coding for a *T. annulata* sporozoite surface antigen (SPAG-1) involved in invasion of cells has been isolated, expressed in *E. coli* (Williamson *et al.* 1989), and found to contain VGVAPG (Hall *et al.* 1992). Thus the elastin receptor is a candidate ligand for sporozoite binding.

Using these novel MoAbs, bovine monocyte/macrophage cells from PBM were characterized, their susceptibility to sporozoite infection assessed, and the subsequent phenotype of the infected cells examined.

Characteristics of peripheral blood monocyte populations

Peripheral blood mononuclear cells (PBM) from Fresian cows were obtained by Ficoll-Hypaque separation (Spooner *et al.* 1989). *T. annulata* does not infect T cells (Spooner *et al.* 1989, Glass *et al.* 1989), and preliminary studies showed that $CD4^+$ and $CD8^+$ T cells are not stained by BCZ, VPM65, IL-A109 or IL-A24 (results not shown). In order to enrich cells of interest in PBM, T cells were removed by lysis with MoAbs CC8 (CD4) and CC63 (CD8) (Howard *et al.* 1991), and rabbit complement (Seralab) according to the method of Teale *et al.* (1986). The resulting T $cell^-$ population was enriched for B cells and monocytes.

T cell-PBM were stained by indirect immunofluorescence and analysed with a FACScan essentially as described previously (Glass & Spooner 1990). Double staining was performed by first incubating with both MoAbs (of different isotypes) followed by appropriate fluorescent secondary reagents: Goat anti mouse (GAM) IgM-FITC; GAM IgG-PE (Sigma, Poole, UK); GAM IgG_1-FITC; GAM IgG_{2a}-PE (Seralab).

The size of the populations stained by the MoAbs was determined by the cells' forward scatter (FSC) upon the FACScan. The majority of IL-$A109^+$ monocytes were small monocytes (mean FSC = 33; Figure 1a) whereas IL-$A24^+$ monocytes were large (mean FSC = 77; Figure 1b). VPM65 recognized both IL-$A109^+$ and IL-$A24^+$ cells. MACS sorted $VPM65^+$ cells (see below) contained three distinct populations in terms of size—small, intermediate and large monocytes respectively (see Figure 1d). Phenotyping of these cells confirmed that three distinct phenotypes were present IL-$A109^+$/IL-$A24^-$ (small); IL-$A109^+$/IL-$A24^+$ (intermediate); IL-$A109^-$/IL-$A24^+$ (large). It is likely that the small IL-$A109^+$ monocytes are immature, becoming larger and IL-$A24^+$ upon activation and maturation.

BCZ stained approximately 16% of T cell depleted PBM, and the majority of these cells (82%) had a distinctive low FSC (mean FSC = 31; Figure 1c) similar to IL-$A109^+$ cells. Double staining with BCZ and macrophage markers revealed that this population was identical to the IL-$A109^+$/IL-$A24^-$ cells described above. The remainder of the BCZ^+ cells were contained in both IL-$A24^+$ populations. B cells were negative for BCZ (results not shown). Thus the elastin receptor is mainly expressed on a discrete population of monocytes and not T or B cells in peripheral blood.

Purification and infection of monocytes

The populations identified by BCZ, VPM65 and IL-A109 were purified using magnetic cell sorting (MACS sorting: see Miltenyi *et al.* 1990 for a review of this method), of T $cell^-$ PBM. The cells were labelled using either VPM65 or BCZ at 1/100 dilution of ascitic fluid, followed by the appropriate MACS bead conjugate (e.g. rat anti-mouse IgG1). The magnetic cells were run through the column twice, and the purity of the separated cells was always > 90%, and usually > 95%. Insufficient IL-A109 was available for MACS sorting. Therefore IL-$A109^+$ monocytes were produced by negative selection in the following way: B cells and T cells were removed by MACS sorting using IL-A30, CC15, CC8 and CC63 (anti sIgM, WC1, CD4 and CD8 respectively. Howard *et al.* 1991). The resulting pure monocytes were subsequently MACS sorted using IL-A24. The IL-$A24^-$ population was 100% IL-$A109^+$ and is referred to in the text as IL-$A109^+$.

The positive and negative cell populations obtained were infected with sporozoites of *T. annulata* (Gharb & Ankara strain) (Brown 1987) in $\log_3$ dilutions, and infection was assessed as previously described (Spooner *et al.* 1989). Frequencies of cells which were transformed were calculated according to Lefkovits & Waldmann (1979) and Brett, Kingston & Colston (1987). Statistically significant differences between cell populations were calculated using analysis of variance. Transformation frequencies of the sorted cells are detailed in Table 1.

Although T cell depleted PBM (T $cell^-$) were not significantly more infectable than PBM ($P < 0{\cdot}1$),

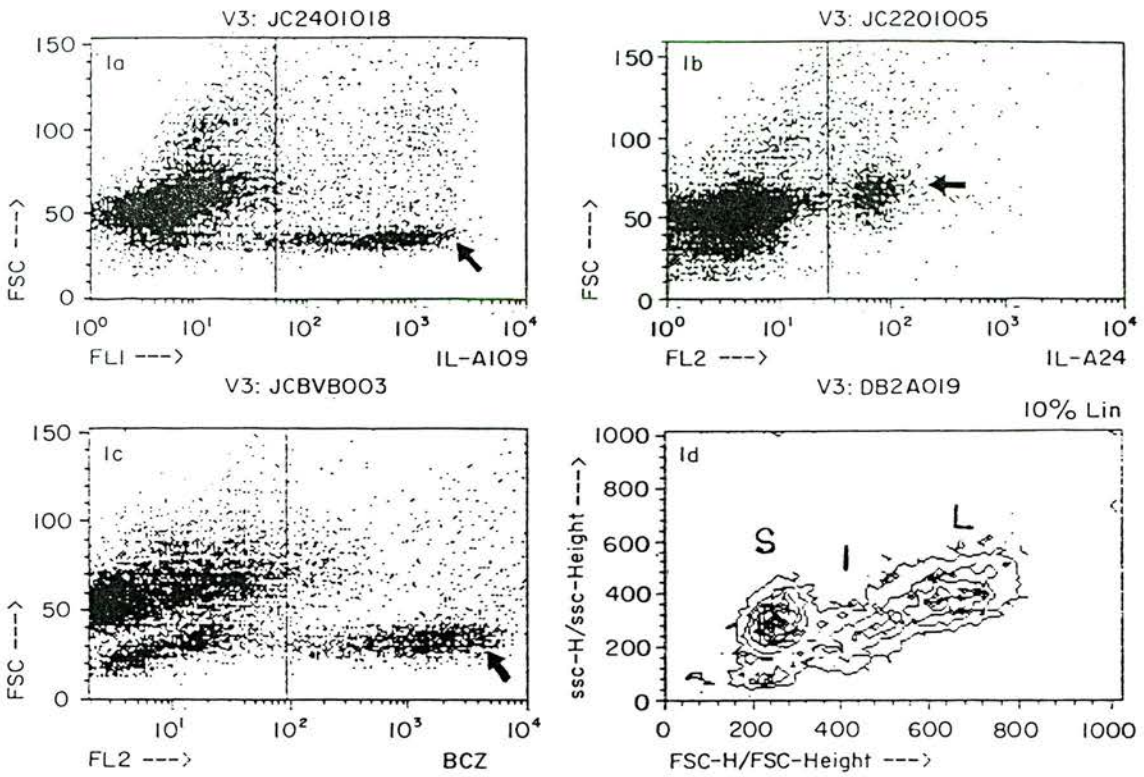

Figure 1 FACS analysis of T cell depleted PBM and CD14$^+$ MACS sorted cells. a–c: Dot plots of T cell depleted PBM stained with a—IL-A109. The majority of cells (arrow) have a low forward scatter (FSC). Mean FSC = 33. b—IL-A24. Stained cells (arrow) have a much larger mean FSC (77) than IL-A109$^+$ cells. c—BCZ. The majority of BCZ$^+$ cells (arrow) have a similar mean FSC (31) to IL-A109. Positive/negative regions are set upon negative controls. d—FSC *v.* SSC contour plot of VPM65$^+$ MACS sorted cells showing small (S), intermediate (I) and large (L) cells.

removing T cells did substantially increase the precursor frequency of infectable cells. Both BCZ$^+$ and BCZ$^-$ cells were highly infectable with *T. annulata*. Thus although the elastin receptor may be a ligand for sporozoite attachment it certaintly is not the only ligand. Sporozoites efficiently infected CD14$^+$ cells, IL-A24$^+$ monocytes and IL-A24$^-$ monocytes but not CD14$^-$ cells (primarily B cells). Taken together, these results confirm the parasite's marked preference for bovine monocytes. We have previously shown that IL-A24$^+$ cells are major targets for *T. annulata* with a very high infection frequency (1:9 within an individual experiment) (Glass *et al.* 1989). The results in this paper show that *T. annulata* preferentially infects these mature monocytes, although it also efficiently infects the more immature IL-A109$^+$ monocytes. Many monocytes within bovine lymph nodes are IL-A109$^+$ (J.Campbell, S.Howie, E.Glass, manuscript in preparation). As the draining lymph node is a major site of parasite development *in vivo* the demonstration of cells within lymph nodes which are potentially infectable with sporozoites is an important finding.

FACS analysis of sporozoite infected cell lines

Cell lines generated from the above cell subpopulations were phenotyped once sufficient infected cells had grown—usually within two weeks. All cells lines were from individual 200 μl wells but not necessarily clonal.

All cell lines produced from *in vitro* infections had the phenotype 100% IL-A24$^+$ and MHC class II$^+$. These results are similar to those reported previously (Spooner *et al.* 1989, Glass *et al.* 1989). Thus even the cell lines derived from the IL-A109$^+$ population had become IL-A24$^+$. Only one of three IL-A109$^+$ cell lines examined contained any IL-A109$^+$/IL-A24$^+$ cells (approximately 25%). Within ten days of first being analysed, this line became IL-A109$^-$. All infected cells were found to be completely negative for CD14 despite this marker being expressed on all monocytes and the CD14$^+$ cells being $>95\%$ VPM65$^+$ when infected. In addition, four *T. annulata* infected cell lines derived previously from *in vitro* infection of PBM had similar phenotypes (results not shown). None of the lines expressed CD14 as

Table 1 Frequency of cells transformed by *T. annulata* as determined by limiting dilution analysis

Experiment	Cell population	Frequency of transformed cells	*P* value	Parasite
1	PBM	1:1055	a	Gharb
	T cell$^-$	1:297	a	
	BCZ$^-$	1:38	b	
	BCZ$^+$	1:20	ND	
2	PBM	1:771	a	Ankara
	CD14$^+$	1:116	b	
	CD14$^-$	1:6897	a	
3	PBM	1:330	a	Gharb
	IL-A24$^+$ monocytes	1:16	b	
	IL-A109$^+$ monocytes	1:52	a	

[1]Within each experiment the same letter denotes no significant difference between frequencies; different letters denote significant differences between frequencies ($P < 0{\cdot}05$).

detected by VPM65. Low numbers of IL-A109$^+$ and BCZ$^+$ cells were detected in two of the cell lines (11% and 18%) and this expression was only seen at low passage. Thus it appears that whatever the initial phenotype of the monocytes infected, infected cells become IL-A24$^+$, IL-A109$^-$, BCZ$^-$ and VPM65$^-$.

Since expression of the determinant recognised by IL-A109 and elastin receptors appears to be restricted to immature monocytes, this evidence suggests that sporozoite infection and subsequent transformation may induce differentiation to a specific phenotype similar to mature monocytes with concomitant loss of 'immature' markers. Alternatively, since the infected cells were not clonal, IL-A24$^+$ cells may be the only cells transformed, subsequently out growing all others. We favour the former, as the loss of CD14 expression shows that sporozoite infection does induce phenotypic change. As IL-A109$^+$ cells were produced by removing IL-A24$^+$ cells, infection cannot be attributed to IL-A109$^+$/IL-A24$^+$ cells. In addition, the 12% of IL-A24$^+$ cells in PBM (see Figure 1b) gave a precursor frequency of 1:330, the precursor frequency of 1:52 observed in the IL-A109$^+$ cells would require an extremely high number of contaminating IL-A24$^+$ cells.

The loss of particular markers may alter the function of parasitized monocytes. For example the loss of FcR may affect the cell's ability to take up antigen and the loss of CD14 may alter the ability of parasitized cells to respond to activation through the LPS receptor pathway. Indeed, we have previously shown that parasitism by *T. annulata* alters antigen presenting function (Glass & Spooner 1990). We are currently investigating some of these phenotypic changes as they may play an important role in the pathogenesis and outcome of the disease.

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Theileria annulata: Altered Gene Expression and Clonal Selection during Continuous *in Vitro* Culture

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SUTHERLAND, I. A., SHIELS, B. R., JACKSON, L., BROWN, D. J., BROWN, C. G. D., AND PRESTON, P. M. 1996. *Theileria annulata*: Altered gene expression and clonal selection during continuous *in vitro* culture. *Experimental Parasitology* **83,** 125–133. Kept in continuous *in vitro* culture, the protozoan parasite ***Theileria annulata*** gradually loses virulence when inoculated into cattle. These attenuated cell lines form the basis of the *in vitro* live vaccines which have been used successfully to control tropical theileriosis in several endemic regions. In the study reported here, events occurring during *in vitro* culture of an Indian (Hisar) cell line, which may be associated with the reduction in virulence, have been investigated. Hybridization with two polymorphic DNA probes following Southern blotting showed that selection of particular parasite genotypes occurs very rapidly with culture; a novel hybridization pattern is observed with both probes after 50–100 passages *in vitro*. In addition to this selection process, immunofluorescence studies using a monoclonal antibody which specifically recognizes virulent *T. annulata* revealed alterations in antibody reactivity following *in vitro* culture. This loss of reactivity was observed in three cloned cell lines derived from the early, virulent Hisar line and implies that phenotypic changes resulting from alterations to parasite gene expression are taking place during the attenuation process. When considered with the results from *in vivo* infections with serial passages of this cell line, it can be proposed that both altered gene expression and selection may be involved in the loss of pathogenicity of *T. annulata* during continuous *in vitro* culture. © 1996 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Theileria annulata*; attenuation; selection; gene expression.

INTRODUCTION

The apicomplexan protozoan parasite *Theileria annulata* is the causative agent of tropical theileriosis, an important constraint on cattle production in Asia, north Africa, and southern Europe (Purnell 1978). Transmission occurs via feeding ticks, which inoculate the infective sporozoite stage of the parasite. Sporozoites invade mononuclear leucocytes which subsequently become immortalized to divide in synchrony with the parasite macroschizonts (Hulliger 1965; Jura *et al.* 1983). Further development of the parasites into merozoites results in the destruction of the host leucocytes (Melhorn and Schein 1984) and invasion of the red blood cells, followed by differentiation into the tick-infective piroplasm stage (Conrad *et al.* 1985).

The ability of the parasites to immortalize host mononuclear cells makes it possible to grow *T. annulata* in continuous *in vitro* culture without the addition of external growth factors (Brown 1980) This continuous passage results in attenuation of the pathogenicity of the disease following parenteral administration of infected mononuclear cells to cattle (Pipano 1981). The observation that these attenuated cell lines continue to afford protection from heterologous challenge has been used in the development of live attenuated vaccines (Subramanian *et al.* 1986). This method of vaccination, however, has drawbacks, not least in the length and expense of the development period and the neces-

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sity to develop vaccine cell lines for each geographical area, due to restrictions on the movement and administration of bovine material.

While the process of attenuation is well established and documented in *T. annulata,* little is known of the molecular mechanisms involved. In other apicomplexan protozoa, both the selection of avirulent subpopulations and marked genomic rearrangement have been reported as resulting in a reduction of virulence. In *Babesia bovis,* a live attenuated vaccine was obtained following rapid syringe passage in splenectomised calves. This is known to be associated with the progressive enrichment of a minor, avirulent subpopulation present at low levels in the original isolate (Cowman *et al.* 1984; Carson *et al.* 1990) and it is assumed that the selection process favours rapidly growing subpopulations (Carson *et al.* 1990). In *Plasmodium berghei,* however, large chromosomal deletions occur following continuous *in vivo* asexual multiplication which have a profound effect on cytoadherence and gametocytogenesis of the parasites (Janse *et al.* 1992). As this would result in reduced pathogenicity of the parasites, it is possible that such alterations lead to attenuation. In *T. annulata,* while no evidence has been presented indicating an association between genetic alterations in the population with loss of virulence, observations of the selection of subpopulations are available. It has been demonstrated that *T. annulata* cell lines contained a number of isotypes of glucose phosphate isomerase (GPI) and that both the parent lines following continuous *in vitro* culture and cloned cell lines derived from the original population contained a single isotype of the enzyme (Melrose *et al.* 1991).

It may be hypothesised, however, if selection of avirulent subpopulations is occurring in *T. annulata,* that this may not be entirely responsible for attenuation. The observation that cell lines continue to lose virulence and eventually infectivity when cultured over long periods of time (Preston, unpublished data) suggests the involvement of a further process, such as altered gene expression, in the loss of virulence. No evidence for altered gene expression has been available in *T. annulata.* A reduction in the level of production of metalloproteases by infected cells following culture has been reported (Baylis *et al.* 1992). It was not shown, however, whether this reduction was due to population selection or alterations in the control of parasite or host cell gene expression.

In this study, different passages of an Indian (Hisar) cell line were compared using indirect immunofluorescence and Southern blotting. Alterations associated with *in vitro* culture were then correlated with data generated following *in vivo* infection with different passage numbers of this cell line in an attempt to define the molecular and cellular events linked to the attenuation of this parasite.

Materials and Methods

Lymphoblastoid cell lines. The *T. annulata* Hisar S45 cell line used in these experiments was derived from the peripheral blood of calves infected with an Indian (Hisar) cell line as described by Brown (1980). Cell lines were maintained in continuous *in vitro* culture at 37°C, 5% CO_2 in RPMI 1640 (Gibco) containing 10% foetal calf serum (FCS) (Gibco) and 2 m*M* glutamine. 100 μg/ml streptomycin, 100 μg/ml kanamycin, 100 units/ml penicillin (all Sigma) and buffered with 2 m*M* Hepes, pH 7.2. Cloned cell lines were derived from passage 4 of the parent cell line by limiting dilution. Cells were serially diluted in 96-well flat-bottomed plates with conditioned medium removed from the parent cell culture. Wells in which growth occurred but which were bordered by empty wells were then maintained *in vitro.* To confirm that the lines were indeed clonal, GPI patterns were compared with those of the parent cell line (Melrose *et al.* 1991). Following infection of calves with serial passages of Hisar S45, cell lines were established from infected cattle as above (Brown 1980).

Assessment of pathogenicity in cattle. Passages 5, 50, and 128 of Hisar S45 were used to infect groups of Hereford/Freisan cattle of various ages. Each calf was inoculated by syringe with 5×10^6 macroschizont-infected cells subcutaneously in the right shoulder. Blood samples were taken at regular intervals and examined for the presence of macroschizonts and piroplasms in the peripheral blood. Rectal temperatures were taken daily to determine the onset of clinical disease. Cell lines were established from infected animals and used in immunofluorescent studies.

Analysis of restriction patterns. Southern blotting was performed using standard protocols (Southern 1975) following *Eco*RI digestion. Macroschizont DNA from serial passages of the Hisar S45 parent cell line, from early passages of clones 3, 21, 54, and 66 and from passages 5 and 25 of clones 18 and 49 was compared following hybridization

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with two polymorphic cDNA probes. Probe Tams 117 recognizes the gene encoding a major internal merozolte antigen while Tams 30 recognizes the gene encoding a 30-kDa merozoite surface antigen (Shiels *et al.* 1994). To determine whether any novel restriction pattern observed was present in a mixed Hisar infection, piroplasm DNA from a *T. annulata* Hisar-infected cow was included in the analysis.

Monoclonal antibodies. Monoclonal antibody (mAb) EU106 was prepared using a standard protocol (Pearson *et al.* 1980) following intraperitoneal inoculation of mice with Hisar S45 passage 3, three times at 11-day intervals (5×10^6 cells). Four days after the final boost, spleen cells from the mice were fused with NSO myeloma cells. Resulting hybridomas were selected in hypoxanthine/aminopterin/thymidine (HAT) medium. Culture supernatants of antibody producing hybrids were collected and stored at −70°C until required.

Indirect fluorescent antibody test (IFAT). Fixed cell preparations were prepared using standard protocols (Shiels *et al.* 1986). Cells were washed twice in phosphate-buffered saline (PBS), pH 7.2, and fixed with 3.7% paraformaldehyde in PBS at a density of 5×10^7 cells/ml on ice for 10 min. Cells were then washed three times in PBS, spotted onto multiwell slides, and air dried at 37°C for 2 hr. To test for reactivity, mAb EU106 was added in 10-μl volumes to wells. Following a 30-min incubation at ambient temperature, slides were washed three times in PBS. Fluorescein–isothyocyanate (FITC)-conjugated sheep anti-mouse IgG (SAPU) diluted 1:40 with PBS was then added to each well and the slides were incubated in the dark at room temperature for 30 min. Following three washes in PBS, slides were mounted in 25% glycerol in PBS and examined by fluorescence microscopy (Leitz Dialux 2000).

Results

Assessment of pathogenicity in cattle. Passages 5, 50, and 128 of the Hisar S45 cell line were used to infect groups of cattle, followed by monitoring of the blood for the appearance of macroschizonts and piroplasms. Those calves inoculated with passage 5 developed severe clinical reactions. Two of the four calves in this group were humanely destroyed. None of the cattle inoculated with either passage 50 or passage 128 of Hisar S45 developed severe symptoms (Preston, unpublished data). The measurement of the time to appearance of piroplasms in the peripheral blood was found to be a good indicator of virulence reduction of the cell line following passage *in vitro*. In calves inoculated with passage 50, the mean time to detection of piroplasms was 16 days, while the figure for those calves given passage 128 was 17.75 days (Table I). No significant lengthening of the time to detection of macroschizonts was observed between the passages, although two out of four calves inoculated with passage 128 did not produce detectable levels of macroschizonts in the bloodstream. In those calves given passage 5, piroplasms were detected in the bloodstream of each 7 days postinfection.

TABLE I

Period to the Detection of Macroschizonts and Piroplasms in the Peripheral Blood of Cattle Infected with Serial Passages (5, 50, and 128) of *T. annulata* Hisar S45.

			Time to detection (days)	
Cell line	Passage No.	CALF No.	MACROSCH.	PIROS.
Hisar S45	Passage 5	413	14	7
		61	16	7
		62	16	7
		55	16	7
	passage 50	35	17	17
		40	14	17
		42	14	14
		45	14	16
	passage 128	260	16	14
		261	19	19
		263	—	19
		265	—	19

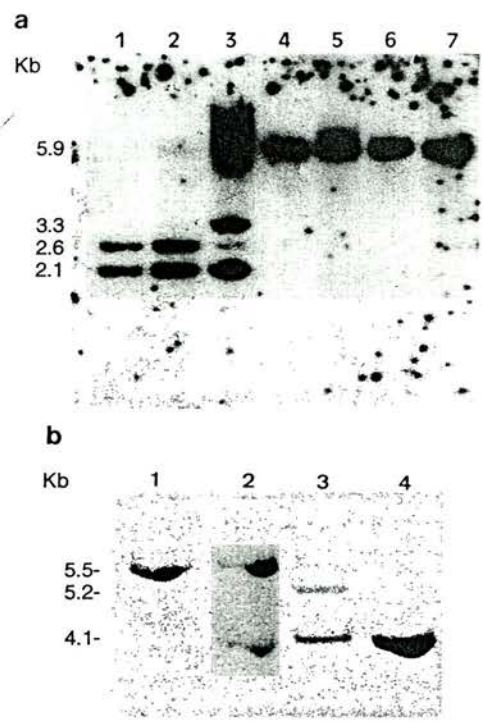

FIG. 1. (a) Restriction pattern of serial passages and clones of Hisar S45 and piroplasm DNA from a mixed Hisar infection following Southern blotting and hybridisation with cDNA probe Tamr 117. 1, S45 passage 5; 2, S45 passage 12; 3, S45 passage 25; 4, S45 passage 50; 5, S45 passage 80; 6, S45 passage 100; 7, S45 passage 128. (b) Restriction pattern of serial passages and clones of of Hisar S45 and piroplasm DNA from a mixed Hisar infection following Southern blotting and hybridisation with cDNA probe Tams 30. 1, S45 passage 5; 2, S45 passage 25; 3, S45 passage 50; 4, S45 passage 128.

Analysis of restriction patterns. The comparison of restriction patterns following hybridization with both of the polymorphic cDNA probes showed that significant alterations took place during *in vitro* passage of Hisar S45. Following hybridization with Tamr117, the early, virulent passage of the cell line displayed two restriction fragments of 21 and 2.6 kb. This pattern was altered by passage 12 to three fragments of 2.1, 2.6, and 5.9 kb and by passage 50 to a single fragment of 5.9 kb. This novel pattern was maintained up to passage 128 (Fig. 1a). An alteration in banding pattern was also observed following hybridization with Tams30. From a single visible band of 5.5 kb at passage 4, the pattern was altered to two bands of 5.5 and 4.1 kb by passage 12, to three bands of 5.5, 5.2, and 4.1 kb by passage 50, and to a single band of 4.1 kb by passage 128 (Fig. 1b). As it has been established that parasite populations derived from cattle consist of a mixture of subpopulations (Melrose *et al.* 1991), it was decided not only to confirm the heterogeneity of the population of the early, virulent parental cell line, but also to attempt to isolate clones with a restriction pattern similar to the later passages of this cell line. Furthermore, cloned cell lines would be used to determine whether alterations in restriction patterns occurred in cloned cell lines as well as in the parent population. Of the clones examined, each had a single restriction fragment of either 2.1 or 2.6 kb following hybridisation with Tamr117, with the exception of clone 18, which had a single fragment of 3.3 kb. The two smaller fragment sizes are represented in the early passage of the parent cell line while passage 12 of the parent line contains a fragment of 3.3 kb. Clone 3, passage 4, had a single fragment of 2.1 kb, while clones 21, 49, and 52 (passage 4) had a single fragment of 2.6 kb. Passages 25 of clones 18 and 49 were also examined for alterations in restriction patterns with culture. Clone 18 had a single band of 3.3 kb at both passage numbers; however, at passage 25, the single fragment of 2.6 kb seen in clone 49 at passage 4 had altered in size to 5.9 kb (Fig. 2a). A similar alteration of fragment size in clone 49 was also observed following hybridisation with Tams30, in this case from a single band of 5.5 kb to one of 4.1 kb. Clone 18 had a single fragment of 4.1 kb after both passage 4 and passage 25 *in vitro* (Fig. 2b).

As none of the clones examined were observed to have a restriction fragment of 5.9 kb following hybridisation with Tamr117 at the earliest passage examined, the possibility existed that this fragment represented a novel rearrangement due to long-term *in vitro* culture and which may be associated with the observed loss in virulence. To determine whether this was the case, piroplasm DNA derived from cattle infected with a mixed Hisar infection was examined to determine whether this fragment was

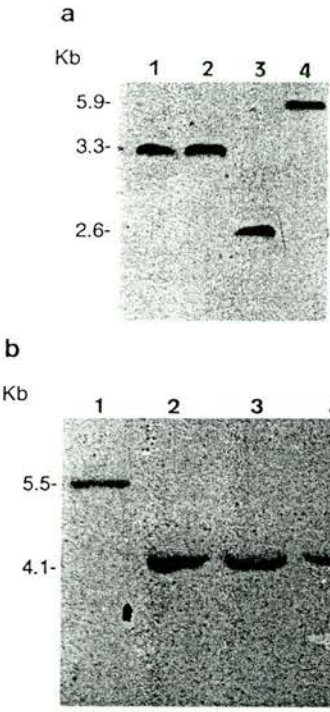

FIG. 2. (a) Restriction pattern of clones 18 and 49 (passages 4 and 25) following Southern blotting and hybridisation with cDNA probe Tamr 117. 1, Clone 18, passage 4; 2, Clone 18, passage 25; 3, Clone 49, passage 4; 4, Clone 49, passage 25. (b) Restriction pattern of clones 18 and 49 (passages 5 and 25) following Southern blotting and hybridisation with cDNA probe Tams 30. 1, Clone 49, passage 5; 2, Clone 49, passage 25; 3, Clone 18, passage 5; 4, Clone 18, passage 25.

in fact due to culture conditions or preexisted in an *in vitro* parasite population. Southern blotting showed that the RFLP of 5.9 kb associated with macroschizont cultures following long-term passage was represented in piroplasm DNA following hybridisation with Tamr117 (Fig. 3) and was therefore unlikely to be a novel rearrangement.

Indirect fluorescent antibody tests. Monoclonal EU106 was used on fixed slides of serial passages of the parent Hisar S45 cell line, and also on serial passages of three clones derived from the early passage of the parent line. Results presented in Table II are expressed as a percentage of cells staining for parasite schizonts in each preparation. In the early passage of

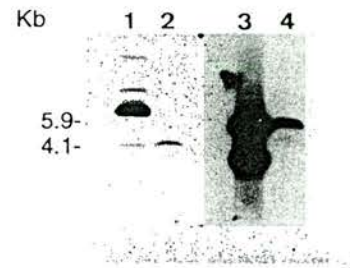

FIG. 3. Comparison of restriction size of passage 128 of Hisar S45 with piroplasm DNA from a mixed Hisar infection following hybridization with either Tams 30 or Tamr 117. 1, Piroplasm Tams 30; 2, S45 p128 Tams 30; 3, Piroplasm Tamr 117; 4, S45 p128 Tamr 117.

the parent line, however, 100% of schizonts showed positive, strong fluorescence (Fig. 4). Following culture, this reactivity was progressively lost until no fluorescing schizonts were observed in preparations of cells at passage 50 (Table II). When serial passages of the three clones were examined for reactivity with the antibody, 100% of schizonts were stained in each of the early passages. In clone 18, this reactivity was rapidly lost with passage; no schizonts were stained on slides prepared with passage 10 of the cloned line. Reactivity of

TABLE II

Percentage of Schizonts of Serial Passages of Hisar S45 and Clones Derived from Passage 3 of the Parent Line Stained Positive with mAb EU106 Following IFAT

Cell line	Passage No.	% Positive with EU106
Hisar S45	Passage 5	100
	Passage 25	100
	Passage 50	30
	Passage 75	0
Clone 3	Passage 5	100
	Passage 25	70
	Passage 50	25
	Passage 75	0
Clone 18	Passage 5	100
	Passage 10	0
Clone 54	Passage 5	100
	Passage 25	100
	Passage 50	40
	Passage 75	0

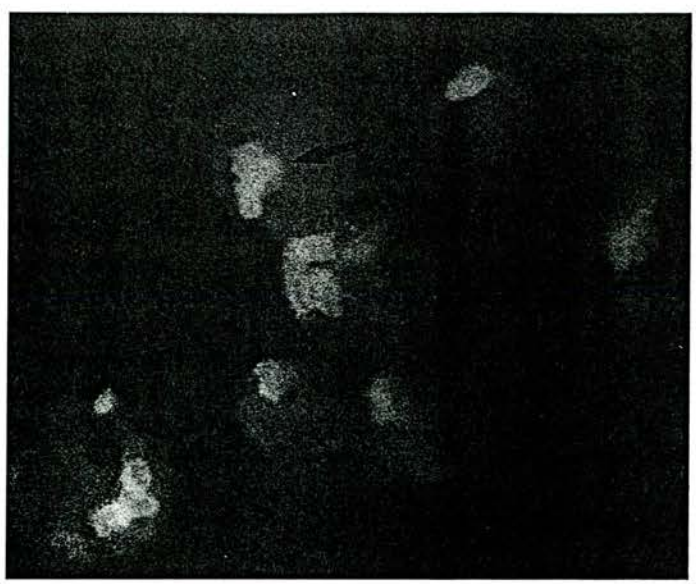

FIG. 4. Fluorescing schizonts of Hisar S45 passage 5 following IFAT using mAb EU106.

clones 3 and 54 with EU106 was also lost with passage, although the process took significantly longer than in clone 18. In both clones, around 50% of schizonts were stained in slides prepared from passage 25 while reactivity was completely lost by passage 50.

DISCUSSION

While the attenuation of *T. annulata* following maintenance in continuous *in vitro* culture is well documented (Brown 1980; Subramanian 1986) it was unknown whether the loss of virulence was due to the selection of preexisting avirulent subpopulations of the parasite or to genetic alteration of a parasite type within the population, presumably followed by the selection and predominance of the altered genotypes. Both alternative mechanisms have been shown to occur in apicomplexan parasites, leading to an actual or proposed reduction in virulence. In *B. bovis,* the gradual enrichment of an avirulent subpopulation has been observed, and this is presumed to be a result of faster growth rate of this parasite type during passage in splenectomised calves (Carson, *et al.* 1990). In *P. berghei,* however, rapid asexual multiplication *in vivo* results in the deletion of regions of the parasite chromosomes, which adversely affects cytoadherence and gametocytogenesis (Janse *et al.* 1992). These faster growing mutants are then selected in culture and are considered to be less virulent. Previous observations of alterations in lines of *T. annulata* kept in continuous *in vitro* culture (Melrose *et al.* 1991; Baylis *et al.* 1992) and the study by Shiels *et al.* (1992), where cloned lines with differing ability to differentiate were represented by distinct genotypes provided circumstantial evidence that selection of parasites with different virulence phenotypes may be taking place, but little indication that any alteration of gene expression may occur. The aim of the present study was therefore to compare serial passages of a cell line to provide evidence that either of these processes was associated with, if not responsible for, attenuation of the parasite.

When serial passages of Hisar S45 were used to infect cattle, it was apparent that virulence was being lost with *in vitro* passage. As the passages used to infect the cattle were available in the laboratory, a good comparison was possible between the amelioration of pathogenicity and molecular alterations observed *in vitro.*

While detailed clinical measurements of fever and parasitaemia are not reported here, the time to appearance of macroschizonts and piroplasms in the peripheral blood was found to provide a good indication of virulence. It should be noted, however, that in addition to a lengthening of the period to detection of the parasites, 2/4 calves infected with passage 5 developed terminal infections (Table I). While it would be interesting to evaluate the virulence of intermediate passages, the results imply that a major part of the virulence has already been lost by passage 50.

Comparison of the passages of this cell line *in vitro* has demonstrated a number of differences that are correlated with the reduction in virulence observed *in vivo*. Evidence of whether selection and/or alterations in gene expression are responsible for these alterations was also obtained. Following Southern blotting of macroschizont DNA, hybridisation with two polymorphic cDNA probes showed that rapid selection of minor subpopulations occurs during continuous passage of *T. annulata*. The restriction pattern observed following hybridisation with cDNA probe Tamr117, which recognises a gene encoding a merozoite rhoptry protein (Shiels *et al.*, 1994), showed that the initial population of Hisar S45 consisted primarily of two populations with single restriction fragments of 2.1 and 2.6 kb. From the initial two bands, the pattern was altered to show three fragments of 2.1, 2.6, and 5.9 kb by passage 12, four bands of 2.1, 2.6, 3.3, and 5.9 kb by passage 25, and finally a single band of 5.9 kb by passage 50, a pattern maintained at passage 128 (Fig. 1*a*). Similarly, with probe Tams30, which hybridises with a gene encoding a 30-kDa merozoite surface antigen (Shiels *et al.*, 1994), an initial single fragment of 5.5 kb was replaced by two fragments of 5.5 and 4.1 kb by passage 12, three fragments of 5.5, 5.2 and 4.1 kb by passage 50 and a single fragment of 4.1 kb by passage 128 (Fig. 1b). One possible explanation of these alterations is that the novel fragments represent changes to the restriction sites caused by genotypic alterations. However, it was also possible that very minor preexisting subpopulations were being selected during *in vitro* culture. For this reason, a number of cloned cell lines, derived from the early, virulent parent line, were examined by Southern blotting. As expected, the majority of clones contained one restriction fragment of either 2.1 or 2.6 kb following hybridisation with Tamr117 (data not shown). It would appear, therefore, that the virulent cell line primarily consists of two major subpopulations. It is apparent, however, that at least one other subpopulation is present, as clone 18 contains a fragment of 3.3 kb, a band size apparent by passage 12 of the parent cell line. None of the clones examined contained a band of 5.9 kb, corresponding to the fragment observed in the late passage of the parent cell line. This may be due either to the selection of a subpopulation present in the initial population at an undetectable level using the analysis employed, or the production of novel restriction fragments via a mechanism of genetic rearrangement. Examination of serial passages of clones 18 and 49 provided mixed evidence for the possibility that a process other than selection was occurring. While no changes were observed in clone 18 by passage 25 *in vitro (vide infra)*, clone 49 showed an alteration in banding patterns following 25 passages *in vitro*, from a single band of 2.1 kb to one at 5.9 kb, similar to that observed in the later passages of the parent cell line (Fig. 2a). Recent evidence (Preston, unpublished data) from GPI analysis has, however, suggested that this cell line is not in fact clonal in nature and appears to have contained more than one genotype, at least at the early passage. This does raise the question of why, if more than one parasite genotype was present in the early passages of clone 49, only one band was observed after hybridisation. In addition to the possibility of cross contamination in the laboratory, other possible explanations may arise from the continual adaptation and eventual selection pressure for in vitro growth of one of the original genotypes, either present in a second cell or possibly following multiple infection of a single cell.

Furthermore, when the fragment sizes obtained with macroschizont DNA were compared with DNA derived from piroplasms ob-

tained from an animal infected with Hisar sporozoites of the parental cell line, bands identical in size to the novel late passage fragments observed in macroschizont DNA were detected. As the probability is low that rearrangement would occur to produce fragment sizes which already existed in the Hisar stock, it would appear likely that the results of the comparison of restriction patterns from the serial passages illustrate the rapid selection of a minor subpopulation of parasites present in the early passage of the parent line.

Evidence of rapid selection was also apparently derived from immunofluorescence studies using mAb Eu106. This antibody, which stains both the parasite schizont, as seen by IFAT (Fig. 3), and the surface of the infected host cell, visualised by FACS analysis (Preston, unpublished results), gradually loses reactivity for the Hisar S45 cell line following *in vitro* culture (Table II). Reactivity for the schizont and the host cell are both lost around passage 50, when the process of selection, as seen after hybridisation with Tamr117, is apparently complete, although selection does appear to be still occurring from the results of hybridisation with Tams30. However, while the loss or alteration of this antigen from the parent cell line indicated the selection of a preexisting subpopulation with no reactivity for the antibody could be occurring, similar studies carried out using serial passages of three clones derived from the early passage of the parent cell line established that the process may be more complex. In all three clones examined, reactivity for EU106 was gradually lost during *in vitro* culture from both the parasite schizonts and the surface of the infected cells. In clones 3 and 54, this loss of reactivity progressed on a similar time scale to the loss of staining in the parent line. In clone 18, however, in which the restriction pattern seen with Tamr117 is unaltered by passage 25, reactivity with EU106 is completely lost by passage 10 (Table II). While no biological role has been established for the antigen recognised by EU106, and therefore no causative link established with a loss of virulence, an alteration in gene expression during culture has been identified. As reactivity is also lost from the parent cell line during *in vitro* passage, it may be hypothesised that selection occurs through an advantage in culture, such as increased proliferation, in those parasites displaying alterations in gene expression. Interestingly, it has recently been found that a parasite cloned cell line which shows limited ability to differentiate to the merozoite *in vitro* has a reduced ability to upregulate gene expression in the initial phase of the differentiation process (Shiels *et al.*, 1994), raising the possibility that similar mechanistic events may be occurring in these two systems.

This alteration in gene expression seems to be maintained following the infection of cattle and the transfer of the parasite to a new host background as cell lines derived from those cattle infected with either passage 50 or 128 of Hisar S45 do not react with the antibody (data not shown). While on a genetic basis this is unsurprising, as no sexual recombination has occurred, this observation lends weight to the view that attenuation is primarily a function of alterations in the parasite rather than the host cell. However, it is unclear whether the alteration of these parasites is fixed genetically, and whether the observed alterations are reversed following tick passage, as has been observed with attenuated lines of *B. bovis* (Carson *et al.* 1990)

While these observations have provided evidence that both selection and altered gene expression occur during the continuous *in vitro* culture of *T. annulata,* a number of questions remain. While changes in the cultures have been observed, these seem to occur too rapidly to account for the continued loss of virulence over long periods of time. Also, the direct relationship of these changes and previous observations of reduced differentiation and production of metalloproteases to the loss of pathogenicity is not known. Furthermore, the relationship between genetic alterations and selection remains to be established, although the results presented in this study suggest that parasites which have an altered ability to control gene expression may eventually predominate following continuous *in vitro* culture.

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A non-protective T helper 1 response against the intra-macrophage protozoan *Theileria annulata*

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SUMMARY

Theileria annulata is a protozoan parasite which infects and transforms bovine macrophages. Infected macrophages possess augmented antigen presentation capabilities, as they are able to activate the majority of T cells from unexposed animals. *In vivo*, T cells in the draining lymph node (principal site of parasite development) are activated 'non-specifically' by the parasite. This event is followed by failure of the immune response to control the infection. Protective immune responses against intra-macrophage protozoa are usually mediated by T helper 1 (Th1) T cell responses. Here we examine the cytokine responses made by *T. annulata*-activated T cells. We show that the outcome of *in vitro* activation of T cells by parasitized macrophages is a skewing of their cytokine responses towards preferential expression of interferon-gamma (IFN-γ) mRNA. The *in vitro* response is mirrored during *in vivo* infection, as greatly elevated amounts of IFN-γ protein are found in lymph efferent from infected lymph nodes, while expression of IL-4 mRNA within the node stops. IFN-γ production does not correlate with protection against the parasite, as infected cells flourish during peak IFN-γ production, and only very small amounts of IFN-γ are produced during the effective immune response of an immunized animal. Overproduction of IFN-γ and loss of IL-4 expression are also likely to account for the failure of B cells to reach the light zone of germinal centres, a developmental step which is tightly regulated by cytokines.

Keywords *Theileria annulata* cytokines T cells interferon-gamma

INTRODUCTION

The protozoan parasite of cattle *Theileria annulata* resides in macrophages during the pathogenic macroschizont stage of its life cycle [1–3]. This tick-borne parasite is of particular interest, as European cattle are extremely susceptible to disease caused by the parasite—Tropical Theileriosis—and this proves a serious barrier to improvement of cattle in endemic areas such as North Africa and India. The macroschizont stage of *T. annulata* does not reside in the endocytic system of the macrophage [4], and differs from other well characterized intra-macrophage protozoans such as *Leishmania* and *Toxoplasma* by 'transforming' the parasitized macrophage into continuous cell cycle. The parasite induces an aggressive infection in susceptible cattle, characterized by expansion of parasite-infected cells in the lymph node draining the tick bite site, accompanied by fever, anaemia, cachexia and anoxia [5,6]. The host immune response is apparently unable to contain the rapidly growing parasite, naive susceptible animals do not develop effective immunity, and death from experimental infection often occurs within 2–3 weeks, depending on the dose of parasite [7,8].

Intracellular protozoan parasites which reside in myeloid lineage cells are now recognized to be controlled largely by T helper-1 (Th1) cytokine responses. In particular, resolution of infection with both *Leishmania* spp., *To. gondii* and *Trypanosoma cruzi* depends on the action of IFN-γ, which may either have direct anti-parasite effects or induce activation of the parasitized macrophage to reject the parasite [9–13]. Both anti-*T. annulata* cytotoxic T lymphocytes (CTL) [14,15] and IFN-γ-mediated induction of nitric oxide (NO) production by macrophages [16] can be demonstrated from animals either rendered immune by treatment with the napthoquinone drug Butalex, or immunized with attenuated macroschizont infected cells (CTL responses only). However, *T. annulata*-susceptible animals are unable to produce similar effective responses during primary infections.

The principal candidate mechanism for immune response failure is the augmented T cell-activating capability displayed by *T. annulata*. Macroschizont-infected macrophages are able to activate most peripheral T cells from unexposed animals in a

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contact-dependent mechanism, with IL-1 production by the infected cells implicated in driving T cell activation [17–19]. Although the antigenic element responsible for T cell activation has yet to be identified, the stimulatory element is unlikely to be a mitogen, as the kinetics of T cell responses to infected cells is similar to antigen-stimulated cells [18].

We have recently shown that this altered T cell-activating ability associated with infected cells disrupts normal T cell activation during *in vivo* infection—the vast majority of draining lymph node T cells are activated 'non-specifically' by infected macrophages. This is followed by failure of T cells to enter normal immune response pathways [18]. The clearest evidence to date that this disrupts normal immune mechanisms is the loss of germinal centre morphology in the infected lymph nodes [18].

An understanding of the effects of this early T cell activation by the parasitized macrophages is an essential step towards an understanding of disease pathology, and ultimately the design of new therapies. Here we show that infection of susceptible animals with *T. annulata* sporozoites induces a rapid production of IFN-γ, and this response is not tightly controlled and damped down as found in immune animals. We further show that parasite-activated T cells preferentially produce IFN-γ mRNA. As well as having no inhibitory effect on the growth of infected macrophages, the unregulated IFN-γ response is associated with local failure of B cell responses, with B cells failing to reach the light zone of germinal centres in infected lymph nodes.

MATERIALS AND METHODS

Animals

Peripheral blood for *in vitro* studies was obtained from normal, healthy Friesian or Friesian cross female or castrated male cattle aged 6 months or older. Calves used for lymph node studies were aged 2 months or older (see individual sections below).

Theileria annulata-*infected cell lines*

Theileria annulata macroschizont-infected cell lines (TaCL) (Hissar or Ankara strain [20,21]) from the animals tested were prepared as previously described [17]. Cell lines were used at low passage number (2–20).

Cell preparation

Peripheral blood mononuclear cells (PBMC) were separated using Ficoll–Hypaque (Lymphoprep; Nycomed, Oslo, Norway) as previously described [22]. Complete tissue culture medium was used throughout the experiments [23].

Culture of PBMC

PBMC were cultured with TaCL essentially as described previously [18,19]. Briefly, PBMC were cultured with autologous irradiated (75 Gy) TaCL in 6×10 ml well plates (Life Technologies, Paisley, UK). PBMC (8×10^6/ml):TaCL ratios were either 10:1 or 20:1 (optimum stimulation ratios were determined previously (results not shown)). A total of seven different TaCL from four unrelated cattle were examined. PBMC ($2{\cdot}5 \times 10^6$/ml) were stimulated with 5 μg/ml concanavalin A (Con A; Sigma, Poole, UK) to provide a 'control' population of activated T cells. Stimulated cells were harvested at various times (1–7 days) for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Theileria annulata-*infected lymph node material*

Archival frozen or paraffin-embedded lymph nodes from *T. annulata*-infected animals were used to examine cytokine profiles and for more detailed examination of the previously reported germinal centre breakdown [18]. Animals had been infected with *T. annulata* sporozoites in the shoulder, and nodes excised during the course of infection [18]. Normal prescapular lymph nodes were examined alongside draining (i.e. site of parasite development) and non-draining lymph nodes removed from calves 2, 4, 8 and 10 days post-sporozoite infection. Thin (2·5–3 μm) sections were cut from paraffin blocks and used for immunohistology. Lymph nodes stored at −70°C in sterile OCT medium (BDH-Merck, Lutterworth, UK) were used for RT-PCR analysis (see below). In addition, cells isolated using a tissue homogenizer (Jencons Scientific, Leighton Buzzard, UK) from lymph nodes at the time of excision were assessed for cytokine mRNA.

Immunohistochemistry

Immunohistochemistry using the ABC system was carried out on lymph node sections using MoAb VPM30 (germinal centre B cells [18]), MIB 1 (Ki-67 proliferation antigen [24]) and anti-human CD3 (Dako A452; Dako, Glostrup, Denmark), as previously described [25], with the following modifications: sections to be stained with MoAb MIB 1 were microwave-treated as previously described [24]; sections for use with anti-human CD3 were treated with pronase as previously described [25]. Double staining was carried out by first staining sections with VPM30 or CD3 followed by microwave treatment and staining with MIB 1 (microwave treatment effectively 'kills' residual avidin-biotin or antibody activity from the first round of staining). Staining was visualized using diaminobenzidine (DAB) as a substrate for horseradish peroxidase (HRP) and vector red (Vector Labs, Peterborough, UK) as a substrate for alkaline phosphatase.

Efferent lymph samples

Cryopreserved cell-free bovine efferent lymph samples [27] generated during a previous study (A. K. Nichani *et al.*, manuscript in preparation) were examined for IFN-γ. Briefly, two of the animals were untreated and efferent lymph was collected for 5–6 days to monitor baseline IFN-γ levels. Two animals were infected with 0·1 TE (tick equivalent) of Gharb strain [28] *T. annulata* sporozoites and developed severe clinical theileriosis. The animals were treated with Butalex (2·5 mg/kg body weight; Pitman-Moore, Uxbridge, UK) as soon as feed intake declined, making a full recovery. Efferent lymph was collected for 10 or 11 days post-infection before drug treatment was necessary. One animal which had been rendered immune by infection with sporozoites and treatment with Butalex was re-infected with 2 TE Gharb strain sporozoites and monitored over the same period.

IFN-γ assay

IFN-γ was assayed in efferent lymph using a commercially available ELISA assay (bovine γ-interferon kit; CSL Diagnostics, Victoria, Australia) according to the manufacturer's instructions. Highly positive lymph samples were diluted up to eight times in PBS in order to obtain results within the range of the detection system. Quantification of IFN-γ was performed by titrating known quantities of recombinant bovine IFN-γ diluted in PBS in the ELISA plate alongside lymph samples. (Recombinant IFN-γ, activity 4×10^6 U/mg, kind gift of Dr R. A. Collins, IAH, Compton, UK.)

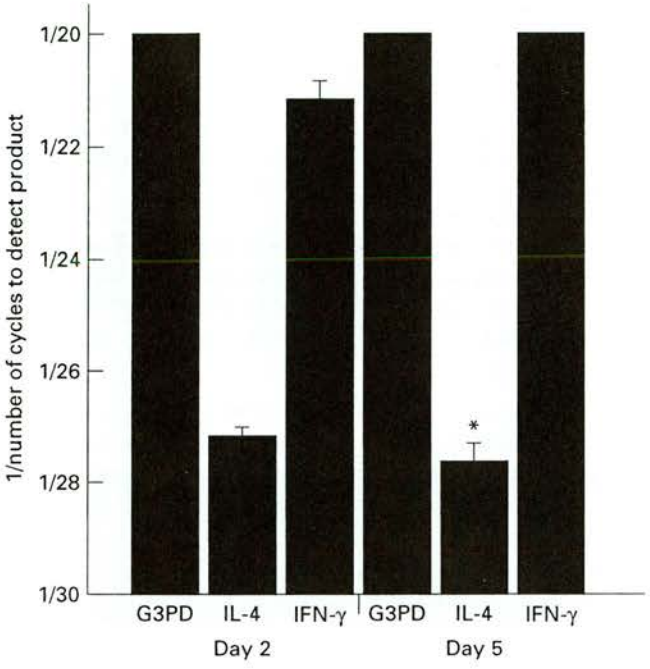


Fig. 1. Reciprocal of mean numbers of polymerase chain reaction (PCR) cycles required to detect glyceraldehyde-3-phosphodehydrogenase (G3PD), IL-4, and IFN-γ cDNA from *Theileria annulata* macroschizont-infected cell line (TaCL)-stimulated peripheral blood mononuclear cells (PBMC) after 2 days ($n = 6$) and 5 days ($n = 7$). Error bars = s.e.m. Where no error bars are present no variance was found between samples. *$n = 6$, one cell line did not induce any detectable IL-4 product in PBMC.

RT-PCR analysis

Although a bovine IFN-γ ELISA was used in this study, this kit is designed for use with serum products, and results with tissue culture samples were found to be unreliable. Also, a bioassay was unavailable for IL-4. As a result, cytokine production was assayed using RT-PCR analysis. *In vitro* stimulated PBMC or lymph node tissue were examined for the levels of expression of mRNA for IL-2, CD25, IL-4 and IFN-γ using a previously described semiquantitative RT-PCR technique [19,26]. Total RNA was isolated using the RNeasy system (Quiagen, Dorking, UK) according to the manufacturer's instructions. *In vitro* stimulated cells ($\geq 10^7$ cells) were washed twice in PBS before RNA isolation, frozen cells were allowed to thaw at 37°C and washed twice in PBS. Frozen lymph nodes were cut from the OCT medium using sterile scalpels and approximately 100 mg of tissue used for RNA isolation.

Total RNA (5 μg) was reverse transcribed using the Superscript system (Life Technologies). cDNA (2 μl) was subjected to different numbers of amplification cycles (20–30) and the products were examined on 2% agarose gels. Comparison of the cycle at which the PCR products became visible was used to assess the relative expression of the cytokine mRNA species [19]. Although this method does not allow direct comparison of mRNA abundance across cytokine primer pairs, it does afford a good estimate of the same mRNA expression after different treatment of cells. The expression of β-actin or glyceraldehyde-3-phosphodehydrogenase (G3PD) was used as an internal control for these reactions. Primer sequences for β-actin have been previously described [26], primer sequences for G3PD were the kind gift of Dr B. Mertens (ILRI, Kenya).

RESULTS

Incubation with T. annulata *macroschizont-infected cells induces a dominant IFN-γ response in peripheral blood T cells*

When T cells are activated by autologous TaCL, peak levels of surface activation marker expression (CD25 and MHC class II) are achieved within 48 h, with maximum proliferation at day 5 [18]. The cytokine profiles of cells responding to TaCL were examined at these timepoints in this study (Figs 1 and 2). All seven TaCL cell lines consistently induced higher levels of IFN-γ mRNA

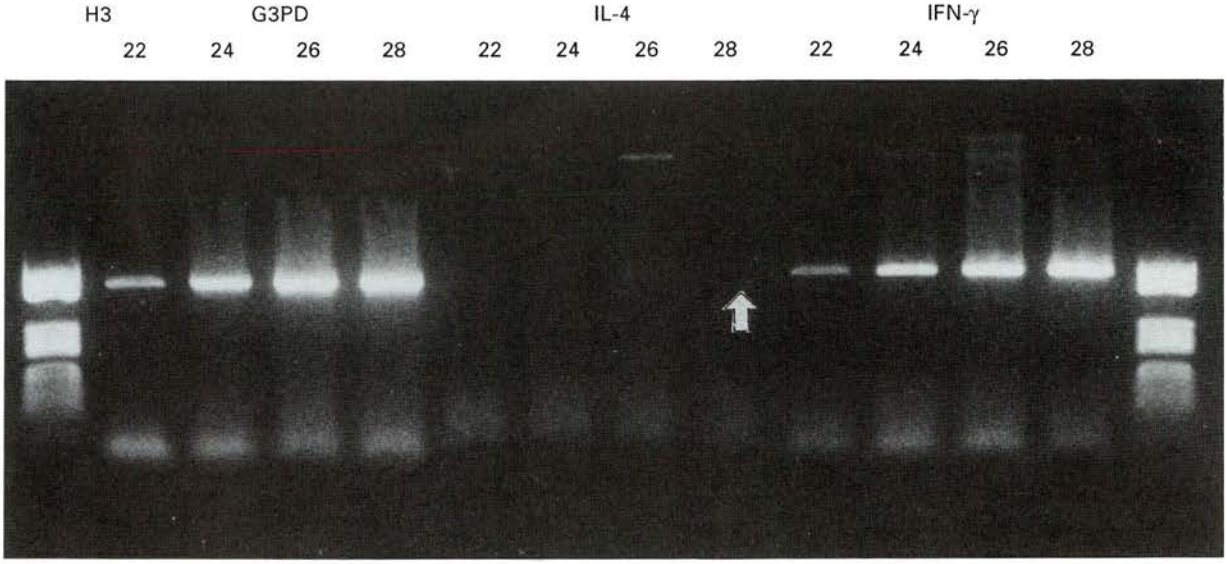


Fig. 2. Limited cycle analysis (cycles 22–28) from a representative animal after 5 days stimulation of peripheral blood mononuclear cells (PBMC) with an autologous *Theileria annulata* macroschizont-infected cell line (TaCL). While glyceraldehyde-3-phosphodehydrogenase (G3PD) and IFN-γ are easily detected after 22 cycles, IL-4 is only faintly detected at cycle 28 (arrowed). H3, *Hae*III markers.

Table 1.

Animal	Treatment	Cycles to detect IL-4	Cycles to detect IFN-γ
12929	TaCL	NP	20
	Con A	22	20
13050	TaCL	28	20
	Con A	22	20
10814	TaCL	28	20
	Con A	20	20

NP, No product detected; TaCL, *Theileria annulata* macroschizont-infected cell line; Con A, concanavalin A.

production than IL-4 in responding PBMC, with the former product easily detectable after 20 cycles in all but one case (22 cycles, 48 h incubation). IL-4 products were never detected until at least 26 cycles of amplification. One cell line did not induce any detectable IL-4 message at day 5.

Working at maximum efficiency, PCR amplification is logarithmic, indicating that each molecule of IFN-γ cDNA, when detected at 20 cycles, required 2^{18} (262 144) amplifications to detect a product. When IL-4 was detected at all, 26–30 cycles were required—2^{24} (4194 304) to 2^{28} (268 435 456) amplifications. IFN-γ cDNA is therefore present in far greater amounts than IL-4 cDNA in TaCL-activated PBMC.

The consistent low abundance of IL-4 mRNA was not due to primer inefficiency, but specifically associated with the activation of PBMC by TaCL. When levels of IL-4 *versus* IFN-γ mRNA production by *T. annulata*-activated PBMC from three animals were compared with Con A-stimulated PBMC at their time of peak proliferation (5 and 3 days, respectively), Con A activation induced very similar levels of IL-4 mRNA and IFN-γ mRNA, whereas incubation with parasite-infected cells always induced far higher levels of IFN-γ mRNA production than IL-4 (Table 1). Simple 'activation' of PBMC therefore does not lead to noticeable differences in levels of IL-4 and IFN-γ mRNA amplified, suggesting that incubation with TaCL is specifically inducing higher IFN-γ production.

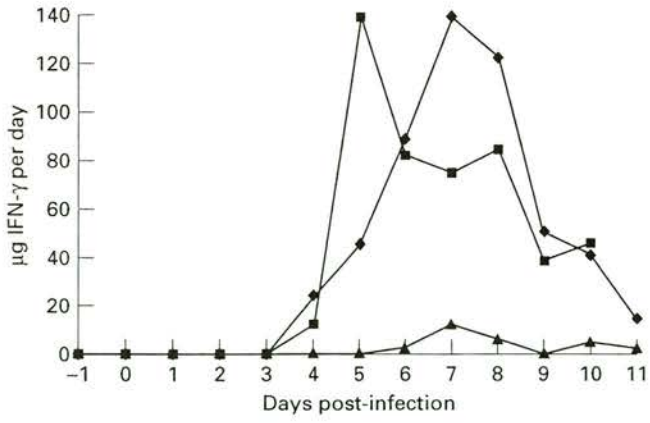
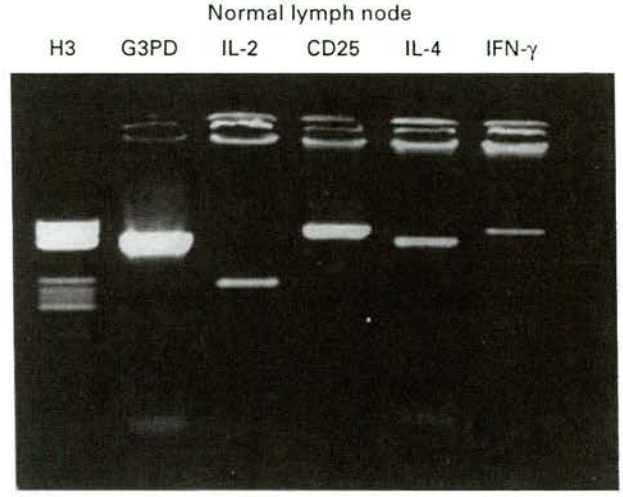


Fig. 3. Levels of IFN-γ protein detected in the efferent lymph of two naive *Theileria annulata*-infected animals and one previously immunized animal. ■, Naive 1; ♦, naive 2; ▲, immune.

In vivo *infection induces an extremely strong Th1 response*

To determine whether high IFN-γ levels were also found during *in vivo* infection, lymph efferent from infected nodes was examined by ELISA. Lymph from uninfected resting lymph nodes showed no



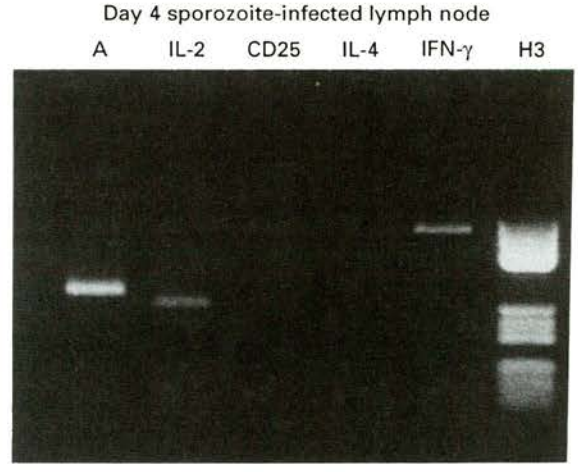


Fig. 4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of bovine lymph nodes. In a normal node, IL-2, CD25, IL-4 and IFN-γ mRNA are present. Four days after infection with *Theileria annulata*, IL-4 and CD25 products can no longer be detected in the draining lymph node. G3PD, glyceraldehyde-3-phosphodehydrogenase. H3, *Hae*III markers.

Fig. 5. Germinal centre within a non-draining lymph node 8 days post-sporozoite infection. Both MIB-1$^+$ dark zone cells (D, stained brown) and VPM30$^+$ light zone B cells (L, stained red) are present. (×400, DAB and vector red.)

Fig. 6. Residual germinal centre within a day 8 sporozoite-infected draining lymph node (same animal as Fig. 5). Despite the continued presence of both a CD3$^+$ T cell zone (T, stained red) and MIB 1$^+$ dark zone (D, stained brown), no VPM30 staining was found in the germinal centre. Mantle zone marked with dotted line. (×650, DAB and vector red.)

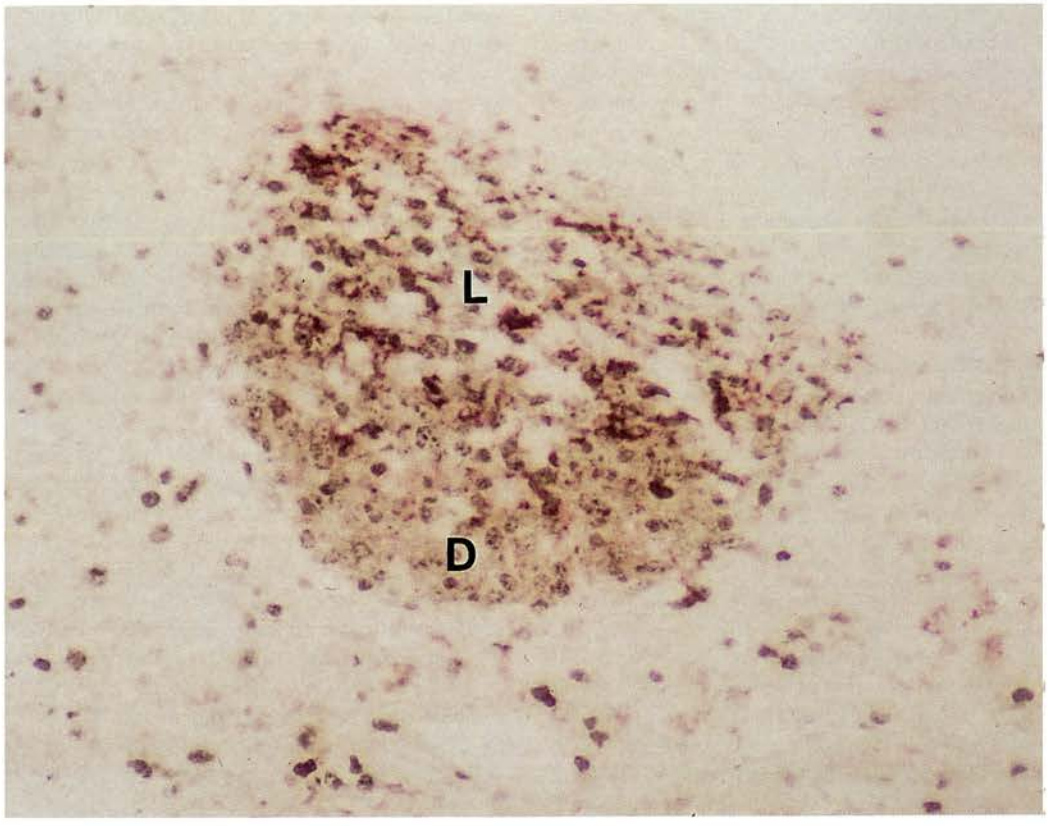


Figure 5

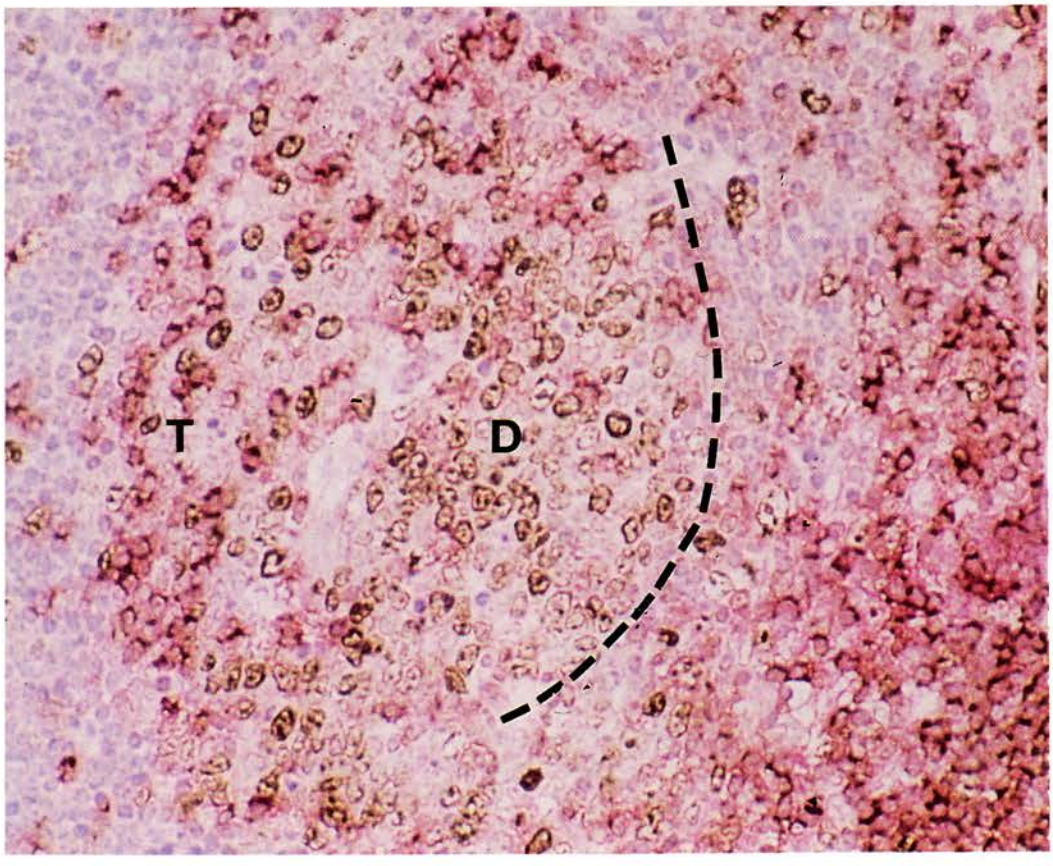


Figure 6

elevation of IFN-γ levels (results not shown). Lymph efferent from nodes draining the site of sporozoite infection in two naive susceptible animals showed very large increases in IFN-γ production (Fig. 3). Over a 6-day period (days 4–9 of infection) a total of $\geq 2 \times 10^6$ U of IFN-γ was detected in the efferent lymph of both naive infected animals. In contrast, lymph efferent from infected draining lymph nodes of an immune animal showed only transient increases in IFN-γ levels (Fig. 3), containing $1{\cdot}1 \times 10^5$ U of the cytokine over the same 6-day period. IFN-γ production in the immune animal appeared to 'shut down' quickly, as the small peak of cytokine production present 7 days post re-infection quickly dropped.

Draining lymph node cytokine mRNA production was assessed by RT-PCR during the peak period of IFN-γ production. Easily detectable in normal lymph nodes, IL-4 mRNA or CD25 mRNA was not detectable in draining lymph node cells within 4 days of sporozoite infection (Fig. 4). This loss of IL-4 mRNA production was found both in fresh preparations from isolated lymph node cells and archival frozen lymph node tissue blocks.

Germinal centre pathology is consistent with a dominant Th1 cytokine response

In vitro, IFN-γ has been demonstrated to interfere with B cell development by inhibiting expression of surface immunoglobulin after the initiation of proliferation [29–31]. We have previously shown that *T. annulata*-infected lymph nodes lose germinal centre (GC) light zone morphology from 8 days post-infection [18]. Here we examined GC morphology in more detail. T cells, proliferating dark zone cells, and VPM30$^+$ light zone B cells were found to be present within GC in normal and non-draining lymph nodes from infected animals (Fig. 5). However, although T cells and proliferating dark zones were found in infected lymph node GC (Fig. 6), VPM30 light zone B cell staining was completely lost from all infected GC by day 8 of infection. The previously reported loss of germinal centre morphology was therefore due to a failure of B cells to pass into the light zone rather than physical destruction by the growing parasite.

DISCUSSION

Theileria annulata-infected macrophages possess augmented antigen presentation capabilities, as they are capable of activating up to 70% of peripheral T cells from unexposed animals [18]. In *T. annulata* infection of susceptible animals, there is evidence of large alterations in immune responses. Infected lymph nodes are characterized by rapidly proliferating infected cells, T cells are primarily activated 'non-specifically' by the parasite, and there is a virtually complete loss of B cell GC. The parasite has therefore developed a survival strategy which not only allows infected cells to flourish, but which also disrupts normal immune responses. Highly successful survival strategies have been developed by intra-macrophage protozoans such as *Leishmania* and *To. gondii* based on inducing Th2 cytokine responses in their hosts, which counteract the Th1 responses which can effectively clear infections [9–13]. In this study we have examined cytokine responses induced by *T. annulata*-mediated activation of T cells both *in vitro* and *in vivo*, and their implications for the induction of effective immune responses. During infection of naive animals leading to severe clinical theileriosis, IFN-γ levels in efferent lymph rose sharply, with approximately 20 times more cytokine produced by naive animals than by an immune animal. The high IFN-γ levels were accompanied by a loss of IL-4 mRNA production in naive animals. Unlike other intra-macrophage parasites such as *Leishmania* spp. and *To. gondii*, the skewing of the host response towards Th1 cytokine production does not confer immunity in *T. annulata* infection. Indeed, the peak time of IFN-γ production (4–9 days) coincides with the time when parasite-associated leuco-proliferation is greatest in infected lymph nodes, accompanied by the appearance of large numbers of macroschizont-infected cells [18]. *Theileria annulata* not only survives in the face of greatly increased IFN-γ production, but appears to actively encourage the induction of a Th1 response. IFN-γ is unlikely to be produced directly by *T. annulata*-infected cells, as mRNA for this cytokine is not usually found in parasitized macrophages [19]. *In vitro* activation of T cells by all *T. annulata*-infected cell lines induced the production of far higher levels of IFN-γ mRNA (at least ×64 more) than IL-4 mRNA.

Most infected draining lymph node T cell activation is 'non-specific' in response to the parasite [18]. As production of IFN-γ in the efferent lymph peaks after large numbers of *T. annulata*-activated T cells are established in the node [18], it seems likely that the parasite is inducing IFN-γ production via non-specific activation of T cells. Although remaining greatly elevated above levels seen from an immune animal, efferent lymph IFN-γ levels do drop partially after 8 or 9 days post-infection. This corresponds to the times when parasite-activated T cells are seen to leave the lymph node in the efferent lymph (A. K. Nichani *et al.*, manuscript in preparation) and further strengthens the link between T cells 'non-specifically' activated by parasitized cells and IFN-γ production.

The specific induction of an IFN-γ-dominated response from *T. annulata*-activated T cells goes some way towards clarifying the impact of the wide range of cytokine mRNAs produced by parasite-infected macrophages upon immune responses to the parasite. Parasitized cells express mRNA for IL-1α, IL-1β, IL-6, IL-10, tumour necrosis factor-alpha (TNF-α) [19] and the p35 and p40 subunits of IL-12 (J. D. M. Campbell and D. J. Brown, unpublished observations). Although a role for IL-1α and IL-6 has been proposed in the induction of T cell proliferation by the infected cells [19], the biological outcomes of the simultaneous production of antagonistic cytokines such as IL-10 and IL-12 [32–35] from the same cell were unknown. The results of this study clearly show that the net influence of *T. annulata*-infected cells on their environment is induction of a Th1 response, both *in vitro* and *in vivo*.

The induction of a Th1 response by *T.annulata*-infected cells via T cells is not likely to be purely driven by the antigen responsible for the T cell activation. When T cells were simply 'turned on' using Con A, the numbers of PCR cycles required to detect IL-4 or IFN-γ were similar, agreeing with other studies which showed that mitogen activation of T cells without the addition of cytokines to skew responses has the potential to induce either Th1 or Th2 responses [36]. This also holds true for 'genuine' antigens—identical *Leishmania* infections in the same inbred strain of mice can lead to either a Th1 or Th2 response, depending on the cytokines used to influence the response [37]. Also, staphylococcal superantigens induce a non-skewed cytokine response in reacting T cells in the absence of other stimuli [38]. It seems most likely that the induction of a dominant Th1 response in T cells responding to activation by *T. annulata* is due to the influence of infected cell-derived cytokines after the initial 'hit' of activation by the parasite. In an immune animal, IFN-γ production appeared to be tightly controlled, as only two small bursts of

cytokine were seen in the efferent lymph. As this control mechanism is evidently absent from naive infected animals, this would again suggest that the *T. annulata*-infected cells are producing factors which strongly promote IFN-γ production and are antagonistic to normal control mechanisms. One obvious candidate for the induction of IFN-γ from responding T cells in naive animals is IL-12 production by infected cells [39], and although mRNA for this cytokine is made by infected cells, further work will be required to determine its significance.

Protection afforded by IFN-γ against pathogens which parasitize macrophages is mediated through activation of the host cell to reject the parasite [9]. This is obviously not a protective mechanism in *T. annulata*, although the NO intermediates associated with killing of intra-macrophage organisms can be detected from infected cells [16]. As *T. annulata* does not parasitize the endocytic system of the macrophage, but exists in discrete membrane-bound vacuoles in the cytoplasm [4], it is likely that it can avoid lysosome-mediated damage. Indeed, it is perhaps unsurprising that *T. annulata* does not appear to be killed by responses designed to activate macrophages, as infection with the parasite induces an activated surface phenotype in the host macrophage [3].

IFN-γ has been demonstrated *in vitro* to interfere with B cell development, after B cells have been induced to proliferate [29]. In this study we demonstrate a similar phenomenon *in vivo*. Once high levels of IFN-γ were produced in infected lymph nodes accompanied by an apparent loss of IL-4 production, germinal centres were found to be 'arrested' at the proliferating dark zone stage, with no B cells entering the light zone. Entering the light zone is dependent upon the expression of antigen-specific immunoglobulin [40]. B cells are particularly sensitive to IFN-γ after initial proliferation, before immunoglobulin is expressed [29], and a lack of IL-4 also inhibits immunoglobulin production [30,31]. It is likely that the failure of B cells to progress to light zones in the infection studied here is due to the action of IFN-γ inhibiting immunoglobulin-dependent selection.

In this study we have shown that, contrary to recognized mechanisms for rejection or survival of similar organisms, the intra-macrophage protozoan *T. annulata* specifically induces the production of greatly elevated levels of IFN-γ. The critical step in the induction of IFN-γ is the 'non-specific' activation of T cells by *T. annulata*-infected macrophages. These findings provide the first evidence of a mechanism for *T. annulata* to avoid destruction by the immune system. Identification of both the antigenic element and infected cell cytokines which induce the non-protective Th1 response in parasite-activated T cells is now essential.

ACKNOWLEDGMENTS

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